A Systematic Study of the Effect of Ultrasound on Food Enzymes and Bioactives in Model and Real Food Systems (Strawberry Puree)

A thesis submitted for the degree of Doctor of Philosophy (PhD)

by

Konstantina Tsikrika

School of Science, Engineering and Technology,
Abertay University.

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Declaration

Candidate's declarations:

I, Konstantina Tsikrika, hereby certify that this thesis submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy (PhD), Abertay University, is wholly my own work unless otherwise referenced or acknowledged. This work has not been submitted for any other qualification at any other academic institution.

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I certify that this is a true and accurate version of the thesis approved by the examiners, and that all relevant ordinance regulations have been fulfilled.

Supervisor ……

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Dedication

I would like to dedicate this work to my parents, Kostas and Chrysoula, and my brother, Dimitris.

Abstract

Ultrasound (US) technology has become of great interest in the food industry, since it can be used as an alternative to conventional thermal methods. However, there is very limited information about the effect of high frequency US on inactivation of food enzymes responsible for food quality degradation, including peroxidase (POD) and polyphenoloxidase (PPO). Thus, the present thesis examines the effect of high frequency US on the activity of commercial horseradish peroxidase (HRP), PPO in mushroom extract, and both enzymes in strawberry puree. Structural alterations of these enzymes after US treatment were studied using time resolved fluorescence. The impact of US treatment on the antioxidant activity, polyphenols, and anthocyanins in strawberry puree samples after treatment and during storage, in comparison with untreated and thermally processed samples was also evaluated. The most effective conditions for complete inactivation of both enzymes were either 378 kHz or 583 kHz at 48 W for 30 min for HRP and 60 min for mushroom PPO. HRP inactivation kinetics upon sonication followed a first order model, whereas those of PPO fitted to the Weibull model. Fluorescence analysis of US treated samples of HRP revealed the removal of the haem from the active centre and the formation of di-tyrosine, possibly mediated by the production of hydrogen peroxide caused by cavitation, while for PPO it was speculated that US caused fragmentation of the tetramer. The POD and PPO activity after sonication of strawberry puree samples was significantly lower than in those untreated throughout storage. Pasteurisation at 90 ^{o}C of strawberry puree inactivated both enzymes however, this treatment had a detrimental effect on anthocyanins, total phenolic content and antioxidant activity of strawberry puree. On the contrary, the application of US enhanced the antioxidant activity, and anthocyanin, and total phenolic content, in strawberry puree samples, as compared to those untreated or pasteurised after treatment and during storage. This study provides a scientific and technological basis to further develop sonication as an alternative to conventional thermal processing of strawberry puree avoiding quality losses of the product.

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Definitions

AA	antioxidant activity
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AS	ascorbic acid
AC	anthocyanin content
BSA	bovine serum albumin
DMSO	dimethyl sulfoxide
DPPH	1,1-diphenyl 2-picrylhydrazyl
EEM	excitation emission matrix
FTIR	Fourier transform infrared spectroscopy
FRAP	ferric ion reducing antioxidant power
FRET	Forster resonance energy transfer
HAT	hydrogen atom transfer
HHP	high hydrostatic pressure
HPH	high pressure homogenisation
HRP	horseradish peroxidase
LOX	lipoxygenase
LY	lycopene
MS	manosonication
MTS	manothermosonication
PAL	phenylalanine ammonia lyase
PG	polygalacturonase
PME	pectin methylesterase
POD	peroxidase
PPO	polyphenoloxidase
PS	photosonication
PVPP	polyvinylpolypyrrolidone
RA	residual activity
SA	salicylic acid
SET	single electron transfer
TAC	total anthocyanin content
TCSPC	time correlated single photon counting

TEAC	Trolox equivalent antioxidant capacity
TPC	total phenolic content
TRES	time resolved emission spectrum
TROLOX	(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
Trp	tryptophan
TS	thermosonication
TT	thermal treatment
Tyr	tyrosine
US	ultrasound
USC	ultrasound with cooling

1 Introduction

The key issue that food scientists encounter is to ameliorate the quality, and concomitantly, to satisfy the increasing demands regarding the quantity of food, in an environmental sustainable way (Burchi, Fanzo and Frison, 2011). Recent studies have shown the importance of phytochemicals and antioxidants in human health and nutrition. In fact, it has been proven that phytochemicals present in fruit and vegetables, such as polyphenols, possess antioxidant, anti-inflammatory and anti-mutagenic properties and consequently, these compounds are of interest to both the pharmaceutical and food industries (Duthie, Duthie and Kyle, 2000a; Zafra-Stone *et al.*, 2007; Nishiumi *et al.*, 2011). As conventional food processing may have detrimental effects on certain nutrients and bioactive compounds present in food, and considering the high demand by the consumers for “fresh-like” products, the food industry is continually searching for new methods of processing and preservation that have decreased negative effects not only on the organoleptic properties but also on the nutritional value of food and food products (Kentish and Ashokkumar, 2011). Thus, emerging thermal and non-thermal processes such as irradiation, ohmic heating, microwaves, pulsed electric fields, high hydrostatic pressure and ultrasound (US) treatment have been investigated as alternatives to traditional processing methods.

US technology has attracted interest in the food and pharmaceutical industries, since it can be used for extraction of bioactive compounds and as a food preservation technique. Therefore, the impact of US on the inactivation of enzymes which are responsible for the deterioration of fruits and vegetables as well on the quality of the respective foods is increasingly studied. However, most of the studies performed have only used frequencies in the range between 20-40 kHz. In fact, there has been no systematic study of the effect of higher frequencies of US on food enzymes, including peroxidase (POD) and polyphenoloxidase (PPO) and the related kinetics as well as on bioactives upon US treatments and during storage. These enzymes are involved in enzyme browning reactions in fruits and vegetables causing food quality degradation therefore their inactivation is of great importance to food industry. Hence, the

aim of this project is to investigate the effect of different US parameters (frequencies, amplitudes) and times on POD and PPO activity and on the levels of bioactive compounds and antioxidant activity of strawberry puree parameters in comparison with thermal pasteurisation and unprocessed products. Kinetic studies of POD and PPO activity are assessed since they can provide valuable information about alterations that may occur on the quality parameters during storage. Structural changes of these enzymes are also investigated in order to obtain an insight into the inactivation mechanism of US. An experimental design was performed in order to optimise US conditions and therefore to obtain products with enhanced quality and extended shelf life.

Specifically, the main objectives of this project are:

- Review of literature regarding the impact of US on food enzymes and bioactive compounds (Section 2)
- Evaluation of the effect of a range of ultrasonic frequencies (20, 378, 583, 862, 995, 1144, and 1174 kHz) on POD (Section 3) and PPO (Section 4) activity;
- Investigation of structural changes which may have occurred on POD and PPO upon US treatment to elucidate information about possible US inactivation mechanism (Section 5).
- Determination of the US impact on POD and PPO activity and health-related compounds in strawberry puree, particularly on the changes in antioxidant activity, polyphenols, and anthocyanins before/after US treatment (Section 6);
- Investigation of the effect US on the enzyme activity, bioactive compounds, and antioxidant activity of strawberry puree during cold storage (Section 6).

2 Effect of ultrasound on food enzymes and bioactive compounds; a review

2.1 Introduction

Ultrasound (US) can be defined as sound waves at a frequency above the threshold of human hearing (~20 kHz) (Soria and Villamiel, 2010; Kwiatkowska *et al.*, 2011). It includes low frequency, high energy US in the kHz range and high frequency, low energy US in the MHz range, which is mainly used for diagnostic purposes (Mason, Paniwnyk and Lorimer, 1996). When US propagates through a liquid medium, regions of alternating compression and expansion are created, which leads to the generation of microscopic gas bubbles. The surface of these bubbles grows larger during the expansion cycle resulting in an increase of the gas infusion and their expansion. Finally, the bubbles collapse violently, creating shock waves, and subsequently, an extreme increase in temperatures and pressures inside the collapsing bubbles is caused. The overall process is commonly referred to as acoustic cavitation (Dolatowski, Stadnik and Stasiak, 2007).

Cavitation generates extreme physical and chemical phenomena: mechanical agitation, microjets, shear forces, microstreaming, hot spots and shockwaves are some of the physical forces caused by cavitation. The high temperatures occurring within the cavitation bubbles lead to a variety of chemical reactions including the generation of highly reactive radicals. The generation of cavitation depends on US characteristics (e.g. frequency, intensity), product properties (e.g. viscosity, surface tension) and ambient conditions (e.g. temperature, pressure) (Ashokkumar, 2014).

The classification of US applications is based on the amount of the generated energy, which is characterised by sound power (W), sound intensity ($W \cdot m^{-2}$) or sound energy density ($W \cdot s \cdot m^{-3}$) (Dolatowski, Stadnik and Stasiak, 2007). However, the uses of US regarding food processing are broadly divided into low and high energy. Low energy (low power, low intensity) US has frequencies higher than 100 kHz, at intensities below 1 $W \cdot cm^2$, while high energy (high

power, high intensity) US uses intensities above 1W·cm^{-2} at frequencies 20 – 100 kHz (Kwiatkowska *et al.*, 2011; Awad *et al.*, 2012).

Power US is used to alter the food properties in a physical, mechanical or chemical/biochemical way and it has mainly been applied in food processing, preservation and safety. It has been used for many years to generate emulsions, disrupt cells and disperse aggregated materials and as an alternative to conventional food processing operations for controlling microstructure and modifying textural characteristics of fat products (sonocrystallization), de-foaming, and in modifying the functional properties of different food proteins (Awad *et al.*, 2012). Other uses of power US include inactivation or acceleration of enzymatic activity in order to enhance shelf life and quality of food products, microbial inactivation, freezing, thawing, freeze drying and concentration, and facilitating the extraction of various food and bioactive components (Dolatowski, Stadnik and Stasiak, 2007).

Low energy US is ordinarily applied in the food technology as a non-invasive analytical technique, providing information on physicochemical properties of food during processing and storage to ensure high quality and safety (Awad *et al.*, 2012). Until relatively recently high frequency US was considered to cause little physical or chemical changes to the properties of the substrates, as it is low power and one of its most common applications is its use for composition measurement and as a tool for food quality assurance (Chandrapala *et al.*, 2012). It has also been used for livestock genetic improvement programme and for estimating the raw and fermented composition of meat products, fish and poultry; for the quality control of fresh vegetables and fruits, cheese during processing, commercial cooking oils, bread and cereal products, bulk and emulsified fat based food products, food gels, aerated and frozen foods, as well as for the detection of honey adulteration and assessment of the aggregation state, size and type of protein (Awad *et al.*, 2012). It should be noted that little work has been carried out on the effect of high frequency US on bio-molecules so interaction of such substrates with US, particularly at frequencies between 100-1100 kHz should be treated with some caution.

US applications have been reported to provide better food safety and quality with faster processing times and reduced chemical and physical hazards (Chemat, Zill-e-Huma and Khan, 2011; Awad *et al.*, 2012). Additionally, as acoustic waves are generally considered safe and environmentally friendly and may be performed in either the laboratory setting or in-line, the use of US has a major advantage over other techniques (Dolatowski, Stadnik and Stasiak, 2007; Kentish and Ashokkumar, 2011).

Regarding food processing, US can be applied in three different ways: directly to the material; by the use of a transducer to couple with the food; or through submerging in an ultrasonic bath. Ultrasound can also be combined with other preservation methods such as pressure (manosonication; MS), heat (thermosonication; TS) or with both together (manothermosonication; MTS) in order to be more effective against pathogenic or spoilage microorganisms or miscellaneous enzymes of industrial importance (Bermúdez-Aguirre, Mobbs and Barbosa-Cánovas, 2011; Rastogi, 2011).

Considering the effect of US on food matrixes and the aim of this project a detailed and critical review of recent literature is presented below. In particular the impact of US processing on enzyme activity, and bioactive compounds present in food are reviewed.

2.2 Effect of Ultrasound on Food Enzymes

Enzyme activity can result in the deterioration of food flavours and food colour. Thus, in order to enhance the stability, shelf life and quality of many food products, certain enzymes must be inactivated, or their activity reduced (Jiang *et al.*, 2016). Heat can easily inactivate enzymes, but it may also negatively change some food properties such as flavour, colour or nutritional value. As a result, US has recently become of great interest, as it can be used to increase or inactivate enzymatic activities with minimal adverse effects on food (Awad *et al.*, 2012; Paniwnyk, 2014).

2.2.1 **Activation of Food Enzymes**

Wang *et al.* (2011) examined the effect of US on the activity of alliinase from fresh garlic. Under the optimal conditions (0.5 W/cm^2 and 40 kHz) sonication enhanced thermal stability, whereas it did not influence its optimal temperature and pH. The application of low frequency and mild intensity ultrasound resulted in increase of the alliinase activity by about 47%. It was also noted that under sonication, the enzyme was inactivated by exogenous pyridoxal 5'-phosphate and K^+, but stimulated by Fe^{2+}, although untreated samples showed opposite results. Furthermore, it was found that US could retard or slow down the inhibitory effect of L-cysteine on the enzyme activity.

Proteases are a very important class of food enzymes with many applications in food industry, such as beer chill proofing, meat tenderising, cheese manufacture, flavour development in fermentation, baking, and health products. The effect of 20 kHz US on alcalase was studied using fluorescence spectroscopy and circular dichroism. It was found that US enhanced the activity of alcalase by 6% over the control when applied at 80 W for 4 min. Spectroscopic techniques showed that the US had increased the number of tryptophan residues on the alcalase surface, raised the amount of α-helices by 5.2% and decreased the number of random coils by 13.6% (Ma *et al.*, 2011). Further work by the same group (Ma *et al.*, 2015) examined polygalacturonase (PG), which is one of the most commonly used enzymes during fruit and vegetable processing, with the aim of enhancing enzyme activity, modifying the PG enzyme structure and enlarging the application range. A 21% increase in activity of PG compared to the control was observed using 22 kHz US at 4.5 W mL^{-1} intensity over 15 min and a study of degradation kinetics of hydrolysis reactions using PG indicated that the US somehow altered the structure of the PG and consequently enhanced its catalytic ability. Fluorescence and far-UV spectra indicated that the US irreversibly decreased the number of tryptophan residues on the PG surface and increased the β–sheet conformation.

Tyrosinase (PPO) is of great importance in the food industry due to it having diametrically opposite effects and studies have been conducted to investigate the possibility of ultrasonic enhancement. On the one hand, it is involved in the

browning reaction in fruit and vegetables but on the other hand tyrosinase is needed for the production of certain organoleptic properties in raisins, cocoa and fermented tea leaves. Yu, Zeng and Lu (2013) evaluated the effect of sonication on the activity of tyrosinase using UV-vis spectrometry and Fourier Transform Infrared Spectroscopy (FTIR) analyses and they observed that US resulted in increased activity of tyrosinase compared to the controls. These results suggested that sonication may increase the likelihood of the combination of substrate and enzyme occurring or may enhance the exposure of the active site to the substrate.

Jadhav and Gogate (2014) used a 20 kHz probe-type sonicator to study the effect of US on a standard lipase enzyme. It was found that the optimum activity of the enzyme was obtained with a power of 12.22 W/cm^2 after 9 min of treatment and under these conditions there was a 2-fold increase in activity. Response surface methodology was used to find the optimal conditions, and various thermodynamic functions (ΔE, ΔS, ΔG, and ΔH) were determined using the immobilised enzyme. The results indicated that there was a definite change in the structural conformation of the enzyme due to sonication.

2.2.2 **Inactivation of Food Enzymes**

Although there are some instances where the enzyme stimulation is desirable, the use of US regarding enzyme activity alteration has been mainly focused on inactivation. An interesting review of ultrasound and enzyme inactivation (and activation) by Mawson *et al.* (2011) presents the US parameters and the other factors that can influence the process. The mechanisms of enzyme inactivation, are also mentioned, including speculations such as slight changes in environmental conditions, including temperature, pressure, shear stress, pH and ionic strength; mechanical and chemical effects of cavitation; and strong shear, and microstreaming. According to Paniwnyk (2014) inactivation is thought to occur via a series of different mechanisms such as thermal effects, free radical generation and impairment of substrates. Islam *et al.*, (2014) investigated the effect of 20-100 kHz ultrasound on the inactivation of enzymes and they elaborated upon the background to cavitation in an attempt to explain the mechanism involved. They suggested that US causes alterations on the

secondary and tertiary structures of the enzyme either mechanically due to cavitation or by the production of hydroxyl radicals caused by the fast formation and collapse of cavitating bubbles.

A relatively recent review on the relationship between the US parameters and the enzyme under study by Delgado-Povedano and Luque de Castro (2015) concluded that there was little knowledge of the specific effects of the energy input and the enzyme. They also pointed out the scarcity of information provided on the US-enzyme-treatment parameters under which each piece of research has been developed, and the necessity for giving thorough information on the data and metadata in order to elucidate each piece of research and thus to allow potential comparison of results from different studies.

Rastogi (2011), discussed the advantages of US treatment, combined with heat, pressure or both in inactivating certain food related enzymes. US was effective in inactivating lipoxygenase (LOX), peroxidase (POD), pectin methylesterase (PME), polygalacturonase (PG) and polyphenoloxidase (PPO) as well as endogenous milk enzymes such as alkaline phosphatase, g-glutamyl transpeptidase, lactoperoxidase, protease and lipase. Tiwari, O'Donnell and Cullen, (2009) have reviewed US inactivation of enzymes responsible for deterioration of fruit and vegetable juice as well as enzymes pertinent to milk quality such as PME, PPO, LOX, POD (in fruit and vegetables), lipase and protease (in milk). They reported that US processing had only a slight impact on the quality of orange, guava and strawberry juices whereas it improved cloud value and stability of orange juice during storage. An early study on POD, LOX, and PPO was performed by Lopez *et al.*, (1994) who utilised a patented unit that allowed the introduction of US (20 kHz) under high pressure at elevated temperatures. They observed a synergistic effect existed which meant that the temperature of inactivation of all three enzymes was greatly reduced. The effectiveness of enzyme destruction increased with amplitude of US and higher temperatures and, not surprisingly, static pressure alone did not seem to have much effect. They suggest that this combined treatment might be useful for solving problems posed by thermostable enzymes in milk, juices and other drinks. A similar review accounting the effects of US processing on enzymes in

plant-based products (Shiferaw Terefe, Buckow and Versteeg, 2015) concluded that, generally, US alone was not effective enough to inactivate enzymes (and microorganisms). However, sonication in combination with slightly elevated temperatures and/or higher pressure was capable of inactivating such enzymes as PME, POD, PPO and LOX, while inactivation enhancement depends on a subtle combination of US properties, the enzyme and the suspension medium.

A study on horseradish peroxidase (HRP) stability and reactivation after MTS and heat treatment (Lopez and Burgos, 1995b) showed that reactivation could occur after both treatments. Addition of sucrose or glycerol to the sample mixture resulted in more inactivation of HRP by heat but the enzyme could be reactivated by MTS especially in the presence of glycerol. However, addition of KCl had a slight protective effect against both treatments. Similar results were observed when LOX was subjected to MTS but the physical parameters of pH, KCl, sugars, glycerol and enzyme concentration were varied (Lopez and Burgos, 1995a). Once more, the inactivation of the enzyme is facilitated by higher US amplitude and a decrease in the pH in the range 5.2-8 raises the resistance of the enzyme particularly regarding thermal treatment. The authors discuss the possible mechanisms for inactivation and implicate cavitation and Fenton-type reactions. MTS of lysozyme at different pressures and temperatures uncovered some interesting results (Mañas *et al.*, 2006). The use of an external pressure of 200 kPa and temperatures between 60 and 80 ^{o}C enhanced the inactivating effect of US (20 kHz) on lysozyme. The heat resistance of the enzyme decreased when ovalbumin is added to the buffer. The application of US (20 kHz) at room temperature and normal pressure did not affect the activity of lysozyme however, raising the external pressure and temperature increased the inactivating effect of US. Thus, a MTS at 70 °C for 3.5 min produced a decrease in lysozyme activity by 10-fold, while inactivation kinetics data followed a biphasic model. The second phase of inactivation was associated with the appearance of free sulfhydryl groups.

Abdullah and Chin (2014) have discussed the progress made in the use of TS as a replacement of conventional heat treatment processes. As well as describing the background to US they provide an overview of TS treatment in

fruit juice production (including the effect of US on specific enzymes) and broaden the approach by including use of TS on fruit juice safety (i.e. the lethal effect of TS on microorganisms).

POD inactivation by TS was described by De Gennaro *et al.* (1999) who performed their work at 80 ^{o}C using 20, 40 and 100 kHz US with powers ranging from 0 – 120 W. The combined treatment of US and heat was effective at inactivating peroxidase and depended on the US power per unit area of the tip and the volume of the solution treated. The effect of heat and TS on the inactivation of POD in watercress was studied in the temperature range of 40 – 92.5 ^{o}C by Cruz, Vieira and Silva (2006). The aim of the work was to see if TS could be applied at lower temperatures than thermal blanching and hence improve the quality of the blanched watercress. Using 20 kHz US the authors showed that the POD enzyme system, found in watercress comprised a heat-labile, and a heat-resistant fraction and a biphasic first order model fitted the experimental data of the purely heat blanching processes. The authors suggested that a first-order model appeared to be the best fit for the TS blanching process because the enzyme inactivation was obtained only by the heat-resistant fraction. The use of US results in shorter blanching times at lower temperatures (compared to purely thermal processing) and this provided a product with a higher quality and/or with minimized processing.

Ercan and Soysal (2011) also investigated the combined effect of heat and sonication (23 kHz) on tomato POD. Up to 50% thermal inhibition of POD was achieved at temperatures of 63 – 67 ^{o}C for 3 min, but US treatment was more effective with 100% of POD being inactivated with 50% power for 150s or 75% power for 90s. The regeneration of POD activity was also examined for the samples exposed to different ultrasonic powers. At 15% and 25% US powers, the residual enzyme activity increased linearly, whereas at powers of 40%, 50% and 75% residual enzyme activity increased nonlinearly. No regeneration of activity was observed in the samples where 100% enzyme inactivation was obtained by US. It was also found that US treatment had little impact on the vitamin C content of tomato extract whereas the samples treated by heat showed a significant decrease in vitamin C levels.

Recently Xin *et al.* (2015) investigated the effects of different thermal and TS (20 kHz at 8, 12 and 16 W/cm^2) treatment on argy wormwood leaves with regard to the inactivation of POD and the retention of total chlorophyll. After thermal treatment below 90 ^{o}C a biphasic first order model was observed but at 90 ^{o}C the inactivation followed first order kinetics, however TS resulted in first order inactivation kinetics at all temperatures studied. TS inactivated POD faster and more chlorophyll was retained compared to thermal treatment with the best conditions being 12 W/cm^2 for 1 min at 80 ^{o}C where 93% of POD was inactivated and 97% of chlorophyll retained.

Cheng, Zhang and Adhikari (2013) have reported on the thermal and TS treatments (20 kHz) regarding the inactivation of mushroom PPO in the range of 55 – 75 ^{o}C. They found that the PPO inactivation followed first order kinetics in both cases but TS was more effective and they suggested that US/heat together could be developed further for an alternative to the so-called “hot break” process for mushrooms. Dias *et al.* (2014) investigated the influence of US on soursop juice (*Annona muricata*) using amplitude levels ranging from 20 to 100% of the total input power (500 W) at constant frequency of 19 kHz for different times (2-10 min), in order to examine the effect of US on PPO activity. Longer processing time (>8min) and higher power intensity (>330 W/cm^2) resulted in lower values of PPO. Apple, pear and strawberry purees were subjected to heat, US, and TS to inactivate PPO and the respective kinetics were compared (Sulaiman, Soo, Farid, *et al.*, 2015a). Room temperature US (24 kHz; 35 W) for 10 min gave a significant reduction in PPO for all the fruits and TS and heat showed first order kinetics with PPO in pear being the most resistant to inactivation. Overall, the application of US allowed lower temperature to be used with potential better fruit quality.

US (20 kHz) for 0-10 min on defatted and diluted avocado puree was studied to determine the effect on particle size, colour, viscosity and PPO activity (Bi *et al.*, 2015). Compared to the results of the previous studies, PPO activity greatly increased under all treatment conditions with the dilutions of 1:2, 1:5 and 1:10 (puree to water ratios) showing an increase of 25%, 37% and 188%

respectively. The authors suggest that the enhanced PPO activity might be due to the observed decrease in particle size.

A study on the TS inactivation of PPO and PME in blackberry juice using response surface methodology showed that the enzyme inactivation and antioxidant properties were enhanced after the application of the optimum conditions (50 °C for 17 min) (Cervantes-Elizarrarás *et al.*, 2017). It was also reported that the extent of enzyme inactivation depended on the composition, the initial enzyme concentration and the pH of the juice and PPO inactivation was greater than PME, probably due to the presence of a thermoresistant PME isoenzyme. Similar observations were made by Illera *et al.* (2018) on cloudy apple juice samples subjected to TS. Samples were treated at a constant frequency of 20 kHz, at different power densities (1.09 – 1.57 W/mL) and temperatures (52 – 67 °C). PME exhibited higher resistance to TS than PPO. At 60 ^{o}C no inactivation was reported for PME, even when the highest density applied. On the contrary, PPO was almost inactivated (3% RA) after TS treatment at 67 ^{o}C and power densities higher than 1.15 W/mL. Concerning the inactivation kinetics of PPO, both the first order and the Weibull model gave a good fit. The group also investigated the impact of dissolved gases in the juice on enzyme inactivation by replacing the air dissolved in the juice by nitrogen or carbon dioxide, prior to the thermosonication experiments. Displacement of air by N_2 followed by TS had the best results regarding enzyme inactivation for both PPO and PME where the RA was 5%, and 55% respectively at 60 °C for 20 min of TS. The inactivation kinetics data for both enzymes fitted well both a first order and the Weibull model.

Aadil *et al.*, (2015) studied TS of grapefruit juice and found that treatment at 60 ^{o}C for 60 min decreased the activity of PME, POD and PPO by 91%, 90% and 89% respectively. Similar work on pear juice (Saeeduddin *et al.*, 2015) showed significant ($p<0.05$) inactivation of the same enzymes by use of US at 65 ^{o}C. Studies on POD and PPO using a factorial central design showed that applying US at an intensity of 376 W/cm^2 for 10 min resulted in a significant ($p<0.05$) decrease in POD and PPO and total inactivation of ascorbase peroxidase (Fonteles *et al.*, 2012). High intensity US (19 kHz) processing of pineapple juice

resulted only in 20% decrease in active PPO after exposure to 376 Wcm^2 for 10 min, whereas heat alone (54 ^oC) had no inactivation effect on PPO activity (Costa *et al.*, 2013).

Recently the effect of thermal and US treatment on the inactivation kinetics of polyphenol oxidase (PPO) and peroxidase (POD) in bayberry juice were examined by Cao *et al.* (2018). PPO and POD in bayberry juice were sufficiently inactivated by thermal treatment, US and US with cooling (USC) treatment. However, the inactivation rates of the two enzymes were higher by US treatment than those by USC and thermal, while inactivation kinetics followed a first order model for all treatments. Moreover, POD found to be more heat and US resistant than PPO in bayberry juice. Another recent study investigated PPO inactivation in apple juice upon High Pressure Homogenisation (HPH), and US with or without cooling, as well as their combination. US treatment without cooling resulted in complete PPO inactivation, whereas the other applied treatments had negligible effect on PPO activity. The inactivation effect of US was attributed to *in situ* generated heat, while the acoustic effect was insignificant (Bot *et al.*, 2018).

The effect of US (40 kHz) in conjunction with ascorbic acid (AS) on the browning and PPO activity of fresh-cut apples were studied by dipping the sonicated and untreated apple pieces in 1% AS solution or water (Jang, Kim and Moon, 2009). After treatment, all samples were stored at 4 ^oC for 12 days whereupon it was found that the AS samples had decreased browning and showed inhibition of PPO activity of 46% and 98% respectively compared to the water treated samples. Further work by the same group (Jang and Moon, 2011) investigated the mechanism by which the US/AS acted and they showed that monophenolase, diphenolase and peroxidase were inactivated but treatment with US or AS separately had the opposite effect.

Raviyan, Zhang and Feng (2005) investigated the inactivation of tomato PME using US at various cavitation intensities and at 50, 61, and 72 °C. Compared to the PME thermal inactivation at 61 °C, TS at the same temperature increased the inactivation by 39 to 374-fold, while at 72 °C the increase was 36 to 84-fold, depending upon cavitation intensity. Generally, the inactivation increased with

temperature and cavitation intensity. A strong synergistic effect was observed in the TS tests and this was more pronounced at low temperatures. The inactivation of tomato PME in all treatments exhibited first order kinetics. Wu *et al.* (2008) reported on the effect of TS on quality improvement of tomato juice and compared the use of 24 kHz US at three amplitudes and 60, 65 and 70 ^{o}C to purely heat treatments. They found that TS at 60 and 65 ^{o}C could produce tomato juice with low PME activity while maintaining high viscosity. In a later work, Terefe *et al.* (2009) investigated the kinetics of PME and PG inactivation in tomato juice using TS (20 kHz) at temperatures between 50 and 75 ^{o}C and compared results with thermal treatment at the same temperatures. Thermal inactivation of PG was described by a fractional conversion model where PG1 isozyme was stable though TS inactivation of PG followed first order biphasic kinetics with PG1 and PG2 isozymes being inactivated at different rates. PME inactivation was described as first order for both thermal and TS treatments and both processes were synergistic. TS enhanced the inactivation of PME by 1.5-6 times and PG2 by 2.3-4 times between 60-75 ^{o}C..

Koshani *et al.* (2015) analysed and optimised the conditions required to inactivate PME in sour orange juice (*Citrus aurantium* L.*)* using both thermal and TS processes. A statistical experimental design was used, and the results indicated that use of heat and US alone were both effective, but the optimal process was to use TS (80 W) for 9.8 min at 63 ^{o}C whereby the PME was completely inactivated. TS of lemon PME was carried out with US at 20 kHz and at various temperatures and compared to just thermal treatment. Heating at 50 ^{o}C with US application for 63 min resulted in an 83% decrease in PME compared to 30% decrease with heat alone (Kuldiloke *et al.*, 2007).

The effect of high-energy US on the functional properties of proteins such as solubility, water retention capacity, as well as gelling, foaming, and emulsifying property have been reviewed (Higuera-Barraza *et al.*, 2016). It was concluded that as well as the intensity, frequency and time of US application, factors that would normally influence the properties of proteins such as pH, temperature and ionic strength are all important. Another recent review concerning the effect of US on microbial growth and enzyme activity (Huang *et al.*, 2017) deduced

that sonication can promote or inhibit enzyme activity by modifying the characteristics of enzymes, substrates and the reactions between them as well as providing the optimal conditions for the reactions.

The effect of very high frequency US (2.64 MHz) concerning the inactivation kinetics of POD in citrate buffer has been studied by Grintsevich and Metelitsa (2002). US with a power input of 1W/cm^2, at temperatures ranging from 35.5 to 55 ^{o}C, and enzyme concentrations between 10 -100 nM were investigated. The rate constants of inactivation for the various treatments were determined and were dependent on the temperature and pH. As the concentration of the peroxidase was increased, the rate of total inactivation changed only slightly, but thermal inactivation significantly decreased. Addition of polydisulphides acted as protectors of peroxidase indicating that hydroxyl and hydroperoxy radicals were involved in the inactivation of the enzyme.

2.3 Effect of Ultrasound on Food Bioactive Compounds

Fruit and vegetable juices contain bioactive compounds such as polyphenols, carotenoids and ascorbic acid, known to possess antioxidant, anti-inflammatory and antimicrobial properties, as well as inhibitory activities towards degenerative diseases (Duthie, Duthie and Kyle, 2000b; Zafra-Stone *et al.*, 2007). Furthermore, the presence of these phytochemicals affects the nutritional value and the organoleptic characteristics such as flavour and aroma perception, while phenolics are also very important in the juice and winemaking technology (Tiwari *et al.*, 2010). Thermal pasteurisation, which is the most broadly applied preservation technique, can have a detrimental impact on product quality. Thus, there is a growing interest in the development of more reliable and simpler techniques that could have fewer negative effects on nutrients as well on sensorial properties of fruit and vegetable juices.

There are numerous reviews concerning the effect of US on the bioactive compounds in food. Soria and Villamiel (2010) in their review of the effect of US on technological properties and bioactivity, mention physical effects of US on emulsification ability, solubility and texture as well as describing the industrial scale ultrasonic aided extraction of bioactives. Interestingly, the authors also

include a section on how ultrasonically generated free radicals have been explored to enhance the functionality of some types of food. Rawson *et al.* (2011) have reviewed the effect of US, as well as other thermal and non-thermal processing technologies, on the bioactive content of exotic fruits such as mango, guava, passion fruit, star fruit, rose apple, papaya, kumquat, pineapple, sapodilla, mamey, lychee and longan. The effect of US in combination with heat on the quality and safety of fruit juice have been reviewed (Abdullah and Chin, 2014). The authors concluded that the cavitation effect of US can enhance enzyme inactivation and microbial destruction however, it might also decrease the juice yield, ascorbic acid content and contribute in colour loss. On a similar note, Pingret, Fabiano-Tixier and Chemat (2013) have reviewed the degradation of food during ultrasonic processing. The review considers food products in general but concentrates on high lipid containing foods such as milk and various types of oil, that may be degraded, and their physicochemical properties altered. It was concluded that some products are altered after exposure to US and consequently the quality of the product is compromised. Regarding food products with high lipid content, lipid degradation induced by cavitation is likely to occur. However, limited number of studies try to clarify the specific mechanism.

Recently, Bevilacqua *et al.* (2018) reviewed the effect of US, amongst other non-thermal technologies, on fruit and vegetable juices. They note that cavitation is more efficient in destroying gram-positive bacteria, spores, spherical-shaped, and small round cells. Additionally, they report that low-power US tends to increase bioactive content in food materials due to enhanced extraction of bound pigments resulting from cell wall disruption. However, they also point out that the use of high power levels and prolonged treatment times might cause losses in phytochemicals such as ascorbic acid, lycopene and polyphenols.

Early work on the effect of US on the anthocyanin content of fruit juices has been reviewed and it was concluded that US had a minimal influence on anthocyanin content of fruit juices (Tiwari, O'Donnell and Cullen, 2009a). It was observed that US processing of orange juice had minimal effect on the

deterioration of colour and ascorbic acid (AS) during storage at 10 ^{o}C. They also mention a small increase (1-2%) in the anthocyanin content (AC) of strawberry juice and more specifically in the levels of pelargonidin-3-glucoside, at lower amplitude levels and processing times. Furthermore, they reported that weak US treatment resulted in an increase in the levels of phenolic compounds in red wine. Two related papers by the same group described the effect of US on AC and AS degradation in strawberry juice (Tiwari, O'Donnell and Patras, 2008) and the stability of the same compounds after sonication (Tiwari *et al.*, 2009). The latter paper reported that higher losses were seen at warm (20 ^{o}C) rather than cold (4 ^{o}C) temperatures and the degradation mechanisms of AC and AS were discussed. Later studies concentrated on AC and colour of red grape juice (Tiwari *et al.*, 2010) where different power levels of 20 kHz US were applied. A full factorial experimental design was employed, and it was concluded that sonication did not significantly affect the AC content or the colour of the juice.

The use of both thermal and ultrasonic treatments on the changes in quality of strawberry puree has been reported by Cheng, Zhang and Adhikari (2013). Physicochemical properties, bioactive compounds (ascorbic acid, total phenolic, and anthocyanin content) and antioxidant activity (AA) were investigated. US treated strawberry puree samples maintained or increased the amount of the bioactives and colour compared to those thermally treated. In addition, there was an increase in radical scavenging ability of sonicated samples when the treatment was carried out for a short time. US in the 20 -1000 kHz range was used to break down fruit matrices to extract bioactives such as ellagitannins and AC in crushed red raspberries and to assess AA and TPC. It was found that 986 kHz and 20 kHz both showed an increase in TPC, while 20 kHz increased AA and AC content by 17% and 12 % respectively (Golmohamadi *et al.*, 2013).

When fresh apple juice was treated with 25 kHz US and different quality parameters were measured it was found that US significantly improved AS, cloud value, TPC, AA and Hunter lab colour values as well as providing a decrease in microbial population (Abid *et al.*, 2013). Work by the same group (Abid *et al.,* 2014a) has shown that a combination of US and HHP resulted in

complete inactivation of plate counts, yeasts and moulds and significantly improved TPC, AS, AA and free radical scavenging activity. Similar results were obtained from apple juice samples upon TS treatment with the optimal process being 25 kHz for 10 min at 60 ^{o}C (Abid *et al.*, 2014b). Abid *et al.* (2014c) studied the influence of US treatment on quality characteristics of apple juice such as polyphenolic compounds (chlorogenic acid, caffeic acid, catechin, epicatechin and phloridzin), sugars (fructose, glucose and sucrose), mineral elements (Na, K, Ca, P, Mg, Cu and Zn), total carotenoids, total anthocyanins, viscosity and electrical conductivity. US (25 kHz; amplitude 70%) was applied to fresh apple juice samples for 0, 30 and 60 min at 20 °C. Juice samples treated for 30 min exhibited the highest increase in polyphenol and sugar levels, while the greatest levels of total carotenoids, minerals (Na, K and Ca) as well as the highest increase in viscosity was observed in the samples sonicated for 60 min. On the other hand, Mg and Cu levels appeared to be decreased in sonicated samples, while total anthocyanin and Zn content, as well as electrical conductivity did not show any differences. Başlar and Ertugay (2013) compared US and photosonication (PS) on the TPC and colour of apple juice and found that the loss of total phenolics was around 23% for heat treatment but only 15% for US or PS and there was an increase in colour intensity compared to the other processing technologies which caused bleaching. On the contrary, Sun *et al.* (2015) reported that sonication decreased the levels of TPC, total flavonoid content and chlorogenic acid as well as the AA of fresh apple juice.

The aforementioned work by Abid *et al.* (2014) has been repeated on carrot juice and the results appear to be virtually identical to those obtained from the apple work. The quality of carrot juice after blanching and sonication treatment with 20 kHz indicated that there was a significant increase ($p<0.05$) in total carotenoids, lycopene and lutein in blanched samples but the values increased after blanching followed by sonication treatment. Certain sugars, chlorogenic acid and Na and K decreased upon blanching but increased in all sonicated samples (Jabbar, Abid, Hu, Wu, *et al.*, 2014). An extension of this work indicated that the combined effect of sonication and acidification on carrot juice stored for 18 days at 4 ^{o}C may be used to improve stability of total phenol, total antioxidants, cloud value and AS (Jabbar, Abid, Wu, *et al.*, 2014). Results

using HHP and US confirmed that this treatment also improved the quality of carrot juice and could be utilised as an alternative to blanching (Jabbar, Abid, Hu, Muhammad Hashim, *et al.*, 2014). Much of this group's work is summarised in a publication relating to the potential of TS in carrot juice processing which concludes that such treatment at 60 ^{o}C is a good alternative to purely thermal treatment and can produce carrot juice with improved amounts of bioactive compounds (Jabbar *et al.*, 2015). Changes in bioactive compounds and shelf life of carrot juice induced by TS (24 kHz, at 50 ^{o}C, 54 ^{o}C and 58 ^{o}C for 10 min) treatment have also been reported by Martínez-Flores *et al.* (2015). TS at 58 ^{o}C retained more than 98% of carotenoids, 100% of AS and, interestingly, the levels of phenolic compounds increased in all stored samples. Alternative processing technologies were applied by Caminiti *et al.* (2012) who investigated the effect of pulsed electric fields, UV or high intensity light pulses in conjunction with MTS on the sensory attributes of an orange and carrot juice blend. They found that Hunter lab colour values were increased compared to the controls however, there were no changes in AA, while TPC was significantly decreased. On the other hand, sensory analysis showed that flavour was adversely affected by all the non-thermal processes as compared to pasteurised control juice.

Freshly squeezed orange juice was sonicated (for 1, 10, 20 and 30 min at 24 kHz) to evaluate impact on selected physicochemical and antioxidant properties, such as TPC, flavonoids, 1,1-diphenyl 2-picrylhydrazyl (DPPH) radical scavenging activity, total carotenoids, AS, pH, Brix and colour attributes. Sonication of juice for up to 30 min at 43–45 ^{o}C showed enhancement in most of the bioactive compounds compared to controls indicating that sonication at mild temperatures is a suitable technique for orange juice processing whilst maintaining nutritional quality (Guerrouj *et al.*, 2016).

Khandpur and Gogate (2015a) examined the effects of US alone and in combination with UV irradiation on the organoleptic properties of orange juice compared with those of conventional pasteurisation. They reported that samples treated with US or combined treatment had similar sensory characteristics with those untreated. Conversely, the thermally treated samples had less pleasant sensory properties than those untreated. The same group

(Khandpur and Gogate, 2015b) in quite a wide ranging paper, describe their results on the use of US and UV on the nutritional quality of different fruit and vegetable juices such as carrot, orange, sweet lime and spinach when compared to conventional thermal processing at 80 ^{o}C for 10 min. They concluded that the use of US treatment produced fruit and vegetable juices with excellent nutritional properties enhanced shelf life, compared to those thermally treated.

The effect of US on tomato pulp has been investigated by Ercan and Soysal (2011) who reported a significant decrease in AS content as a function of temperature and time whereas US treatment had no significant effect. Anese *et al.* (2013) examined the impact of sonication of tomato pulp on the microstructure and the *in vitro* bio-accessibility of lycopene by using US treatment at a 24 kHz frequency. It was observed that US treatment resulted in loss of tomato cell integrity and in a reduction in the degree of pectin esterification, whereas it enhanced the tomato pulp structure. The latter led to a reduction in lycopene *in vitro* bio-accessibility of the treated tomato pulp. In contrast, total lycopene content was not influenced by sonication, while processing time had a small impact on the release of lycopene from the pulp network. Further work was carried out by Anese *et al.* (2015) on the influence of US processing on the lycopene concentration and bio-accessibility of tomato pulp containing no sunflower oil or increasing amounts (2.5%, 5% and 10%). It was found that neither sonication treatment nor oil addition altered lycopene content straight after the treatment, but a decrease in lycopene levels after the 15th day of storage was observed. US treatment did not affect lycopene *in vitro* bioaccessibility of tomato pulp as compared to untreated samples, whereas oil addition resulted in decrease in lycopene *in vitro* bioaccessibility. All samples exhibited losses in lycopene *in vitro* bioaccessibility during storage and it was mainly attributed to carotenoid degradation.

Alighourchi *et al.* (2013) studied the influence of ultrasound on AC, TPC and AA in juice samples extracted from two pomegranate parts (whole pomegranate and arils alone). Changes in AC of sonicated samples were not statistically significant, compared to the controls. However, they observed a variation

among diglucoside and monoglucoside anthocyanins which was attributed to the separation of sugar from diglucoside anthocyanin structure, degradation of polymeric anthocyanin or co-pigmentation reactions. TPC in juice samples treated for 3-9 min at 100% power was significantly increased as compared to those untreated.

Fonteles *et al.* (2012) used power US to examine the effect on quality parameters of cantaloupe melon juice. Amongst others, they analysed the TPC of the treated samples and it was found that sonication resulted in the decrease in phenolic compounds by 30%. US processing was, however, able to improve and keep juice homogeneity (cloud stability) during 6 weeks at 4 ^{o}C. Freshly squeezed watermelon juice was subjected to TS (20 kHz) using different variables and Hunter lab colour values, lycopene, TPC and AS content were determined. Higher retention of lycopene and AS was noted at low treatment conditions but AA, lycopene, and TPC were decreased significantly at high energy input and longer times leading to the conclusion that TS significantly affects key watermelon juice parameters (Rawson *et al.*, 2011).

Costa *et al.* (2013a) examined the impact of high-intensity US treatment on physicochemical properties of pineapple juice and it was observed that there were no statistically significant differences on the levels of phenolic compounds in the treated samples compared to those untreated. They also reported that sonication increased the stabilisation of the treated juice samples after 42 days of storage compared to those that were non-sonicated. The same group investigated the use of US treated pineapple juice as a substrate to *Lactobacillus casei,* to develop a probiotic drink. Sonicated juice found to be an appropriate substrate for probiotic microorganism cultivation and subsequently, an alternative, non-dairy probiotic drink (Costa *et al.*, 2013b).

Dias *et al.* (2014) investigated the influence of ultrasound on soursop juice quality parameters, such as AS and TPC, through response surface methodology. Higher processing time (>8min) and power intensity (>330 W/cm^2) resulted in an increase of juice temperature, which confirmed the impact of the cavitation phenomenon. The use of high intensities resulted in good retention of phenolic compounds, although the results were not statistically

significant. It was also recorded that the levels of AS were higher in most of the treated samples, compared to those untreated and there was good acceptance of flavour from sensorial testing. Similar results on AS content of soursop nectar treated with TS were reported by Anaya-Esparza *et al.* (2017). They observed up to 90% retention of AS in samples upon TS at 24 kHz (1.4 W/mL) and 54 ^{o}C, while there were not significant changes in quality parameters such as colour and non-enzymatic browning index.

The impact of heat and ultrasound treatment on food ingredients such as AS, AA and TPC of Chokanan mango (*Mangifera indica* L.) juice has been studied by Santhirasegaram, Razali and Somasundram (2013). The samples were thermally treated at 90 °C for 30 and 60s and sonicated (40 kHz; 130 W) for 15, 30 and 60 min at 25 °C. The extractability of carotenoids and polyphenols in sonicated samples and carotenoid and TPC increased significantly compared to the controls. It was also observed that carotenoids remained stable after 15 and 30 min sonication, possibly due to the inactivation of enzymes responsible for degradation of carotenoids caused by cavitation induced shock waves and ultrasonic reaction. In contrast, US treated samples as well as those thermally treated showed a significant decrease in the AS content when compared to those untreated. In later work, it was reported that there was better retention of individual phenolic compounds and an increase in the AA in the US/UV treated samples. Furthermore, sensory evaluation indicated that the non-thermally treated juice was preferred (Santhirasegaram *et al.*, 2015).

Quality assessment of pear juice upon US treatment and commercial pasteurisation processing conditions has been reported by Saeeduddin *et al.* (2015). The latter treatment (95 ^{o}C for 2 min) showed the highest losses of AS, TPC, flavonoids and AA, whereas use of US (20 kHz, 70% amplitude) at 65 ^{o}C for 10 min showed the best retention of these compounds. Similar observations were made by Ordóñez-Santos, Martínez-Girón and Arias-Jaramillo (2017) on Cape gooseberry juice after 10 min sonication at 42 kHz and 30 ^{o}C. They reported increased levels of TPC, and carotenoids, whereas there was a decrease in AS content.

The use of combined microwave-US pasteurisation on sour cherry juice indicated that high temperature in the presence of US led to a significant decrease in AS content. Response surface modelling showed that the optimum conditions were 352 W microwave power, 475 W US power at 50 ^{o}C for 6 min (Samani *et al.*, 2015). Chaikham and Prangthip (2015) evaluated the effect of HHP (300-500 MPa), US (20 kHz) and thermal treatment (50-100 ^{o}C) processing on the phenolic contents and AA of honey from the longan flower. After application of pressure, the quantities of bioactives increased and US had a similar effect but heat treatment at 100 ^{o}C showed the lowest levels of TPC and AA. It was concluded that both HHP and US processing offered an alternative for the preservation of bioactives of longan flower honey without compromising nutritional values. A recent study examined the effect of US (28 kHz, 60 W, 15 min), pulsed light and their combination on the phenolic content and antioxidant activity of blueberry juice, fermented with lactic acid. The combined treatment found to be the most suitable method resulting in significant increase in TPC, total flavonoid concentration, AC, antiradical activity against DPPH scavenging activity, ABTS radical cation scavenging activity, and reducing power capacity in fermented juice samples compared to those untreated (Kwaw *et al.*, 2018).

2.4 Conclusion

Ultrasound has attracted a huge amount of interest in food science and technology as an emerging technology that can complement, or be merged, with traditional techniques. The inactivation of enzymes that may result in the deterioration of food without significantly altering the sensory properties or nutritional value of food, as discussed in this review, exhibit that US can play an important role in the evolution of new value-added food processing techniques. However, there are considerable difficulties when comparing different work mainly because of the terminologies used or lack of information about the applied US conditions. While most of the work reported previously has studied US frequencies around 20-40 kHz there are very few reports on the application of higher frequencies (300-1100 kHz) which potentially offer additional opportunities to optimise US use in order to provide even greater

enhancements. Future research is needed to determine fully the impact of sonication on the physicochemical properties of food, the effect on bioactives and organoleptic characteristics. These research needs prompted the present work on the evaluation of the impact of high frequency US on food enzyme activity and on bioactive compounds/antioxidant activity.

3 The Effect of Ultrasound on the Activity of Peroxidase

3.1 Introduction

Peroxidase (POD) is an enzyme, ubiquitous in the plant kingdom (Civello *et al.*, 1995), and it constitutes part of the defensive mechanism of plants against a variety of stresses caused by viral, microbial, or fungal infections, salt stress, wounding, or air pollution (Castillo, Penel and Greppin, 1984). It is a haem-containing oxidoreductase, which reduces hydrogen peroxide in the presence of an electron donor in a wide variety of organic and inorganic compounds (Kwak *et al.*, 1995; Veitch, 2004). The catalytic multi-step reaction can be described as follows (Nokthai, Lee and Shank, 2010):

$$\mathrm{POD} + \mathrm{H_2O_2} \rightarrow \mathrm{Compound\ I} + \mathrm{H_2O}$$

$$\mathrm{Compound\ I} + \mathrm{AH_2} \rightarrow \mathrm{Compound\ II} + \mathrm{AH^{\bullet}}$$

$$\mathrm{Compound\ II} + \mathrm{AH_2} \rightarrow \mathrm{POD} + \mathrm{AH^{\bullet}} + \mathrm{H_2O}$$

AH_2 and $AH^{\bullet}$ represent the substrate (hydrogen donor) and its radical (oxidised donor), respectively. A simplified equation for this chemical reaction is the following:

$$\mathrm{H_2O_2} + 2\mathrm{AH_2} \xrightarrow{\mathrm{POD}} 2\mathrm{H_2O} + 2\mathrm{AH^{\bullet}}$$

The most studied POD is horseradish peroxidase (HRP), often used as an indicator of efficient blanching, as it is very heat stable, and relatively simple and inexpensive to assay (Machado and Saraiva, 2002; Połata *et al.*, 2009). HRP has also commercial uses as a component of clinical diagnostic kits and immunoassays therefore extraction of peroxidase from horseradish roots occurs on a relatively large scale (Veitch, 2004).

It has been reported that POD is involved in enzymatic browning since it can oxidise diphenols (López-Serrano and Barceló, 1996; Richard-Forget and Gauillard, 1997). Enzymatic browning is caused by the oxidation of polyphenolic compounds, resulting in the formation of dark brown polymers (López-Serrano and Ros Barceló, 2002). However POD's involvement in browning reaction is dependent on the availability of electron acceptor compounds such as superoxide radicals, hydrogen peroxide or lipid peroxides (Chisari, Barbagallo and Spagna, 2007). Hence, POD's presence, has been associated with

discolouration, and losses in flavour as well in nutritional and consequently market value of food and food products (Fortea *et al.*, 2009). Therefore, the inactivation of POD is a requisite in food industry.

Traditionally, thermal processes have been used for enzyme inactivation (Cruz, Vieira and Silva, 2006); however heat resistant enzymes require high temperatures, which can have detrimental impact on sensory characteristics and certain nutrients of food (Ercan and Soysal, 2011). This, in combination with the increasing demands of the consumers for fresh-like products, was the impetus of food industry to search for alternative methods of food processing and preservation with less detrimental effects (Terefe *et al.*, 2010a). Ultrasound (US) is one of the emerging technologies that has been used as an alternative to conventional thermal processes.

Numerous studies have reported the effect of US on various enzymes, including peroxidase, and have been recently reviewed by Delgado-Povedano and Luque de Castro, (2015). Nevertheless, most of them were performed at low frequency (20 – 40 kHz). Recently Cao *et al.*, (2018) observed POD inactivation in bayberry juice after US treatment at 20 kHz and different amplitudes, while Rojas *et al.*, (2017) reported that the application of 20 kHz, alone or as a pre-treatment, resulted in POD inactivation in green coconut water. Ercan and Soysal, (2011) also found that the application of low frequency US at 23 kHz lead to POD inactivation in tomato, while Fonteles *et al.*, 2012 reported POD inactivation after sonication at 19 kHz in cantaloupe melon juice.

A few studies have reported the combined effect of US with other methods such as heat, pressure, and additives. Thermosonication resulted in HRP inactivation as noted by De Gennaro *et al.*, (1999), while Cruz, Vieira and Silva (2006) observed similar results in watercress POD after thermosonication. Abid *et al.* (2014) described the combined effect of US and HHP on POD inactivation in apple juice, while Jang and Moon (2011) noted that the combination of US and ascorbic acid lead to POD inactivation in fresh-cut apples. Earlier, the application of manothermosonication resulted in HRP inactivation (Lopez *et al.*, 1994).

There is very little information about the effect of higher frequencies of ultrasound on enzymes (Grintsevich and Metelitsa, 2002; Rachinskaya, Karasyova and Metelitza, 2004). In fact, there has not been a systematic study of a range of higher US frequencies regarding HRP inactivation. Hence, the current work aims to investigate the effect of low and high frequency US at different acoustic power levels and times on the inactivation kinetics of commercial HRP, in order to gain a better understanding of the kinetics and mechanism of US in POD inactivation.

Specifically, the objectives are:

- To examine the impact of higher US frequencies (378, 583, 862, 998, 1144 and 1174 kHz) on HRP inactivation kinetics in comparison with that of low frequency (20 kHz) at different acoustic power levels (2.1 – 64 W).
- To evaluate the effect of similar power levels at different frequencies on HRP activity in order to determine the optimum energy input required to inactivate the enzyme.
- To compare the effect of US on HRP inactivation with that of a control thermal treatment.

3.2 Materials and methods

3.2.1 **Chemicals**

Peroxidase from horseradish (HRP, EC 1.11.1.7), potassium phosphate monobasic, potassium phosphate dibasic, hydrogen peroxide, and guaiacol were purchased from Sigma-Aldrich, Gillingham, UK. All chemicals were analytical grade.

3.2.2 **Enzyme solution**

In order to optimise the concentration of the enzyme solution used in the experiments, a standard curve of HRP activity was prepared using concentrations ranging from 0.001 mg/mL to 0.0075 mg/mL (Appendix A, Fig A 1). After plotting the graph, the concentration of 0.005 mg/mL was chosen in order to examine US and thermal effects on HRP activity.

3.2.3 **Ultrasound treatments**

The US equipment used in these experiments was either a Misonix Ultrasonic Liquid Processor operating at 20 kHz (FB705, Fisher Scientific, Pittsburgh, PA, USA) or a Meinhardt Ultraschlltechnik high frequency sonicator (HM8001-2, Leipzig, Germany) with a Meinhardt Power Amplifier (M-11, Leipzig, Germany). The high frequency sonicator is equipped with two transducers: a F701 transducer operating at 378, 995 or 1175 kHz and a F712 transducer operating at 583, 862 or 1144 kHz. The transducer is connected to a glass reaction vessel with a cooling jacket. The high frequency equipment is shown below, in Fig 3.1.

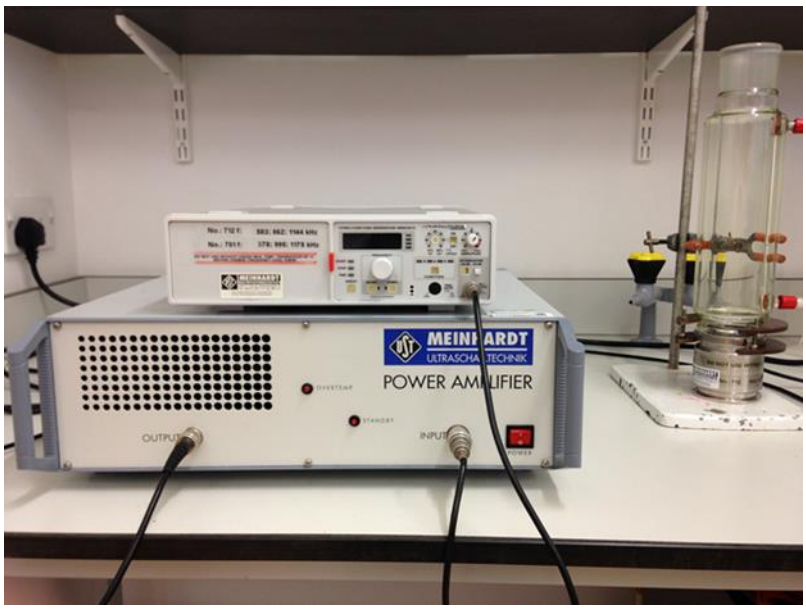


Figure 3.1 The high frequency US equipment consisting of the sonicator, the power amplifier and the transducer connected with a jacketed glass reaction vessel

Various amplitudes corresponding to different acoustic powers were also selected, while the power output (W) was determined by calorimetry. In brief, the change in temperature of a known volume of deionised water was monitored by a thermometer over a certain time and a series of US amplitudes were chosen for the different frequencies used. Calculations were performed using Eq 3.1:

$$\mathrm{Q} = \mathrm{c}\ \mathrm{m}\ \Delta T$$

Equation 3.1

where Q is number of Joules, c the specific heat of water (4.18 J g^{-1} $^{o}C^{-1}$), m mass of water used (g) and ΔT change in temperature (^{o}C) (Milne, Stewart and Bremner, 2013).

The starting temperature for all the ultrasonic experiments was 20 ± 2 °C and the temperature profile was recorded every 5 min. All ultrasound treatments were performed in triplicate for 60 min and samples were withdrawn for analysis after 2, 4, 6, 8, 10, 15, 30, 45, and 60 min of treatment. Table 3.1 shows all the combination of power and frequencies investigated in this study.

Table 3.1 Combinations of frequencies and powers used in sonication treatments

Frequency (kHz)	Power Level (W)					
20	n/a	11	16	35	n/a	n/a
378	3.9	10	17	32	48	n/a
583	2.1	8.9	17	34	48	n/a
862	4.3	9.6	n/a	20	n/a	64
995	4.9	9.4	17	24	n/a	n/a
1144	3.4	8.8	17	n/a	49	n/a
1175	3.4	n/a	15	39	n/a	n/a

All the samples were re-analysed after being kept at 4 °C for 24 h in order to investigate if any re-activation of the enzyme occurred after treatment and/or during storage.

3.2.3.1 Low frequency experiments

The low frequency experiments were performed using a Misonix Ultrasonic Liquid Processor (Fig 3.2) fitted with a 1.3 cm titanium probe operating at 20 kHz in a 4 s pulse on 2 s pulse off mode at different amplitudes, which correspond to 11, 16 and 35 Watts (determined by calorimetry). The probe was immersed in a 400 mL beaker containing 200 mL HRP solution (0.005 mg/mL) with the probe tip positioned 20 mm from the bottom of the beaker. A thermometer was also positioned in the beaker to allow the temperature to be monitored during the sonication.

Figure 3.2 The low frequency US equipment. The processor consists of a horn connected with the probe operating at 20 kHz.

3.2.3.2 High frequency experiments

A standard volume (200 mL) of HRP solution (0.005 mg/mL) was introduced into the glass reaction vessel (62.5 mm internal diameter) and a thermometer was suspended in the reaction liquid in order to monitor the temperature during sonication. The starting temperature for all the ultrasonic experiments was adjusted to 20 ± 2 °C and cooling was applied through the jacketed reactor (wall thickness 5 mm) by use of water pumped through a cryostatic bath (Fisher Scientific, ISOTEMP 4100 Thermostatic, Pittsburgh, PA, USA).

3.2.4 **Thermal treatment (control)**

The effect of heat at 40 (±2) °C was also examined for comparison purposes, considering that the temperature during sonication treatments did not exceed 43 ^{o}C. Glass test tubes containing the enzyme solution (2 mL) were placed in a thermostatic bath previously equilibrated at the specified inactivation temperature. At pre-determined time intervals, three test tubes were taken out of the bath, immersed in an ice bath and then analysed.

3.2.5 **Enzyme assay**

HRP activity (ΔA_{470} min^{-1} g^{-1} FW) was monitored as an increase in optical density due to the oxidation of guaiacol to tetraguaiacol, following a modified method of Castillo, Penel and Greppin, (1984) and previously performed in published work (Andrianos *et al.*, 2016). The complete reaction mixture contained potassium phosphate buffer (100 mM; 1.0 mL; pH 6.1), guaiacol (96 mM; 0.5 mL), hydrogen peroxide (12 mM; 0.5 mL), enzyme solution (0.005 mg mL^{-1}; 0.1 mL), and deionized water (0.4 mL). The enzyme activity was measured at 470 nm in glass cuvettes over a period of 1 min on a UV-Vis spectrophotometer (UV-1650 PC, Shimadzu UK Ltd, Milton Keynes). The percentage of residual activity (RA) of HRP was calculated as below:

$$\text{Residual Activity } (\%) = \frac{\mathrm{A_t}}{A_0} \times 100$$

Equation 3.2

where A_t and A_0 are, respectively, HRP activity after and before the treatment.

3.2.6 **Statistical analysis**

Data analysis was performed using Mixed-Linear model by IBM SPSS Statistics 23 in order to examine if there was an interaction between power, frequency and time at each experiment. A mixed effect model was produced with power (W) as a fixed factor and time (min) and frequency (kHz) as covariates. Variation between replicate samples was treated as a random factor. Specifically, possible interactions between time and frequency, time and power, frequency and power, as well as simultaneous interaction of these three factors were investigated. Linearity between the predicted values and the dependent variable (HRP RA), normality of distribution of residuals, and independence of residuals were also checked, and the plots can be found in Appendix A (Fig A 2 – A 4). Values presented are the mean of experiments done in triplicate and replicated 3 times (n = 9). The values were considered significantly different when $p<0.05$.

3.2.7 **Kinetics of HRP inactivation**

The inactivation rate constant k is estimated by linear regression analysis of the natural logarithm of residual activity versus treatment time. The first order kinetics model (Eq 3.3) was the most suitable to fit the inactivation of HRP.

$$\ln\left(\frac{A_t}{A_0}\right) = -kt$$

Equation 3.3

where A_0 is the initial activity, and A_t is the activity at time t, and k is the inactivation rate constant (min^{-1}) at the frequency/power studied. The calculation of RA and the plots of ln(RA) versus time were performed using Microsoft Excel.

3.3 Results and discussion

3.3.1 **Effect of US on HRP inactivation kinetics**

A wide range of frequencies (20, 378, 583, 862, 995, 1144, and1175 kHz) at different acoustic powers (2.1 – 64 W) were used to evaluate their effect on HRP activity. All sonication treatments resulted in significant ($p<0.05$) decrease in HRP RA after 60 min of treatment, while inactivation followed a first order kinetics model. It should be pointed out that enzyme reactivation did not occur after any treatment.

Fig 3.3 shows the inactivation kinetics curves of HRP when low frequency at 20 kHz was applied at power levels 11, 16, and 35 W. The kinetics of inactivation fitted well in the first-order model with the correlation coefficients (R^2) ranging from 0.993 to 0.998. It can be easily seen that inactivation rate increased with higher ultrasonic power with the inactivation rate constants (k-value) of HRP treated at 20 kHz varying from 6.4×10^{-3} min^{-1} at 11 W, to 7.8×10^{-3} min^{-1} at 16 W, and 20.9×10^{-3} min^{-1} at 35 W. All three treatments significantly ($p<0.05$) decreased HRP activity. HRP RA was recorded 68%, 62% and 28% after 60 min of sonication at 20 kHz and 11 W, 16 W and 35 W, respectively.

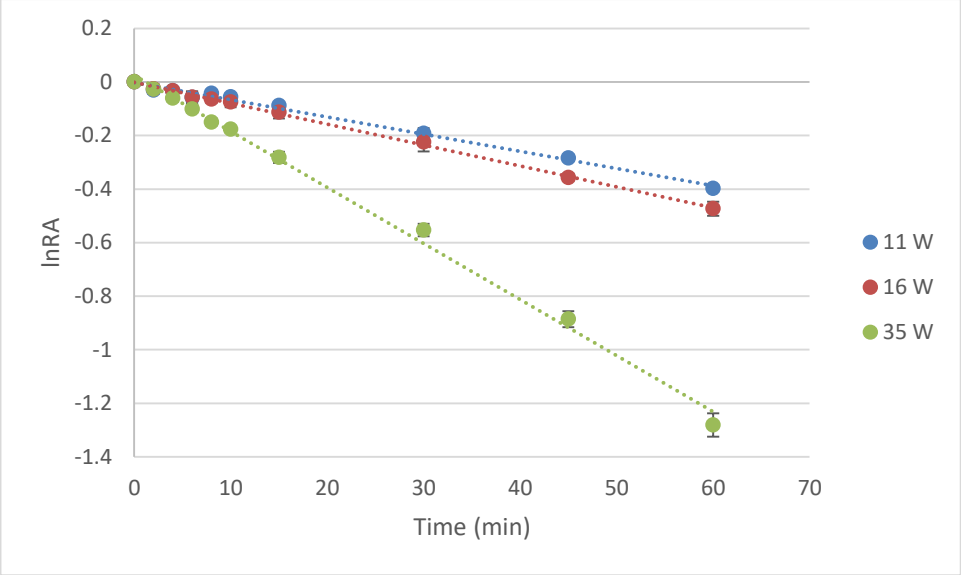


Figure 3.3 Inactivation kinetics curves of HRP treated at low frequency (20 kHz) and different power levels (11 – 35 W). Values presented are the average (n=9) ± STDEV

The effect of high frequency US at 378 kHz and power levels 3.9 W – 48W on HRP inactivation kinetics can be seen in Fig 3.4. Inactivation data followed the first order kinetics model with R^2 values ranging from 0.857 to 0.995. Inactivation rate increased with increasing power input, with k values being recorded as 2.5, 15.6, 31.1, 58, and 138×10^{-3} min^{-1} after sonication at 378 kHz and 3.9, 10, 17, 32, and 48 W respectively. HRP RA decreased significantly ($p<0.05$) and was found to be 86%, 38%, 15%, and 3% after 60 min of US treatment at 378 kHz and 3.9, 10, 17, and 32 W respectively, while 378 kHz at 48 W resulted in complete inactivation of HRP after 45 min of treatment.

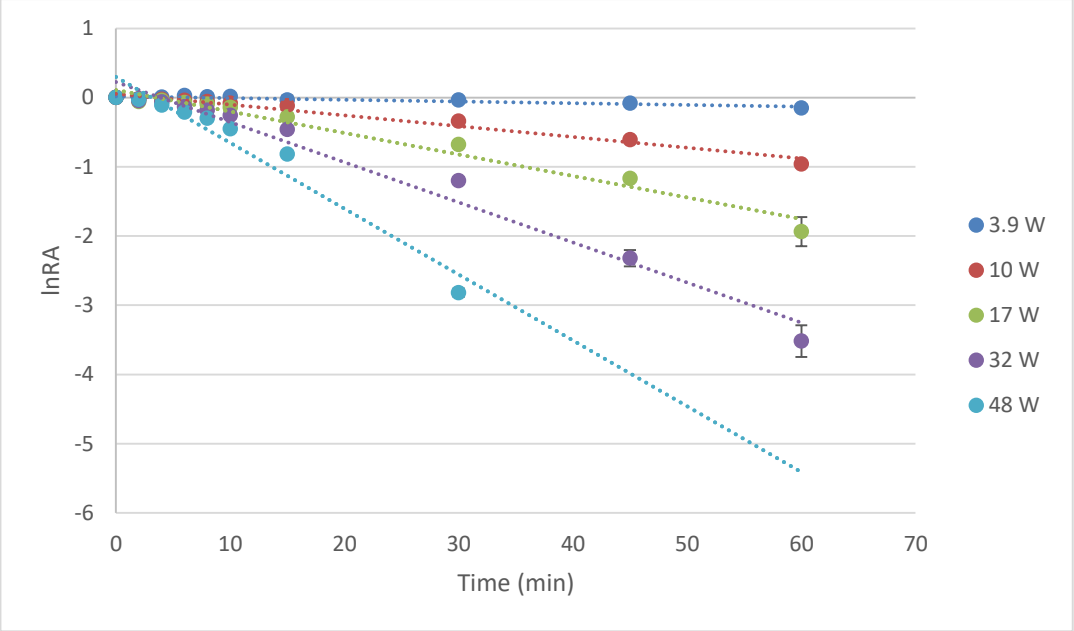


Figure 3.4 Inactivation kinetics curves of HRP at 378 kHz and power levels ranging 3.9 – 48 W. Values presented are means (n=9) ±STDEV.

As shown in Fig 3.5 inactivation kinetics of HRP by US at 583 kHz and 2.1 – 48 W fitted well the first order kinetics model (R^2=0.944-0.999). Values of k constant varied from 4.7, 8.2, 18.4, 59 to 114×10^{-3} min^{-1} after sonication at 583 kHz and 2.1, 8.9, 17, 34, and 48 W, indicating an elevated rate of inactivation with increasing power. Furthermore, the application of 583 kHz at power levels 2.1 – 48 W decreased significantly ($p<0.05$) HRP activity after 60 min of treatment. HRP RA was recorded 79%, 61%, 36%, and 3% after 60 min of US treatment at 583 kHz and 2.1, 8.9, 17, and 34 W respectively, while 583 kHz at 48 W completely inactivated the enzyme after 45 min of processing.

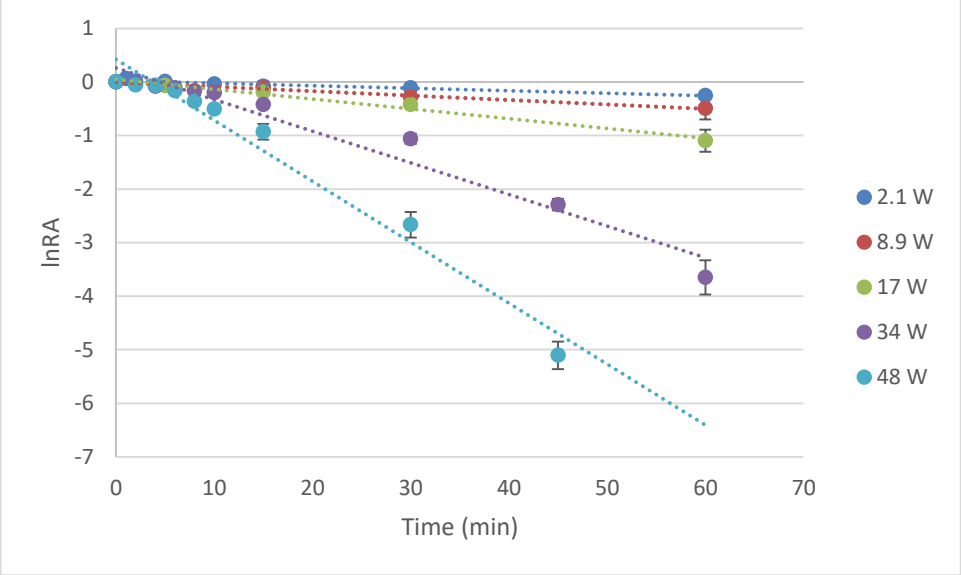


Figure 3.5 Inactivation kinetics curves of HRP at 583 kHz and power levels ranging 2.1 – 48 W. Values presented are means (n=9) ±STDEV.

Fig. 3.6 shows the inactivation kinetics curves of HRP after use of US at 862 kHz and 4.3, 9.6, 20, and 64 W. Inactivation kinetics data fitted the first order model with R^2 values ranging from 0.959 to 0.997. The inactivation rate constant (k) values increased when higher powers were applied, and were found to be 2.2, 5.5, 14.1, and 46.3×10^{-3} min^{-1} after US at 862 kHz and 4.3, 9.6, 20, and 64 W, respectively. HRP RA decreased significantly ($p<0.05$) and was recorded as 90%, 72%, 42%, and 2% after 60 min of the aforementioned conditions.

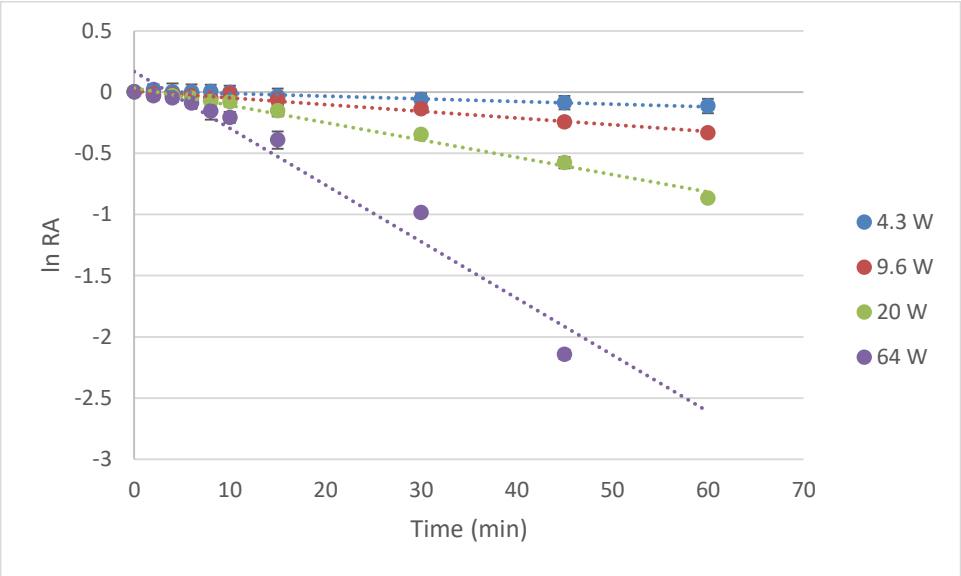


Figure 3.6 Inactivation kinetics curves of HRP at 862 kHz and power levels ranging 4.3 – 64 W. Values presented are means (n=9) ± STDEV.

The impact of US at 995 kHz at power levels 4.9 – 24 W on inactivation kinetics of HRP is shown in Fig 3.7. A first order kinetics model fitted the inactivation data well with R^2 value varying from 0.988 to 0.996. Values of k were 4.2, 6.9, 19.0, and 31.7×10^{-3} min^{-1}, after the application of 995 kHz at those power levels, respectively, suggesting increased inactivation rate with increasing power input. HRP RA was recorded 76%, 65%, 30%, and 13% after 60 min of sonication at 995 kHz and 4.9, 9.4, 17, and 24 W, respectively, with all the data being statistically significant ($p<0.05$).

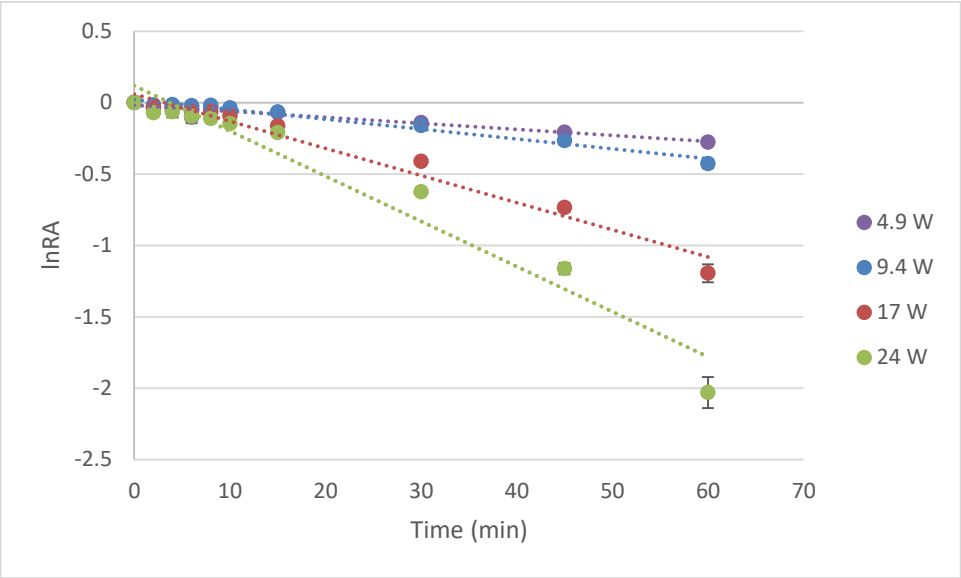


Figure 3.7 Inactivation kinetics curves of HRP at 995 kHz and power levels ranging 4.9 – 24 W. Values presented are means (n=9) ± STDEV.

Inactivation kinetics of HRP by US at 1144 kHz and power levels ranging from 3.4 to 49 W can be seen in Fig 3.8. Inactivation data followed the first order kinetics model with R^2 value ranging from 0.977 to 0.998. Inactivation rate constant (k) value was recorded 3.4, 7.4, 21.1 and 45.0×10^{-3} min^{-1} after these US conditions, respectively, indicating elevated inactivation rate with increasing power. HRP RA decreased significantly ($p<0.05$) after 60 min sonication. HRP RA was found 81%, 64%, 42%, and 6% by the end of the treatment at 1144 kHz and 3.4 W, 8.8 W, 17 W, and 49 W, respectively.

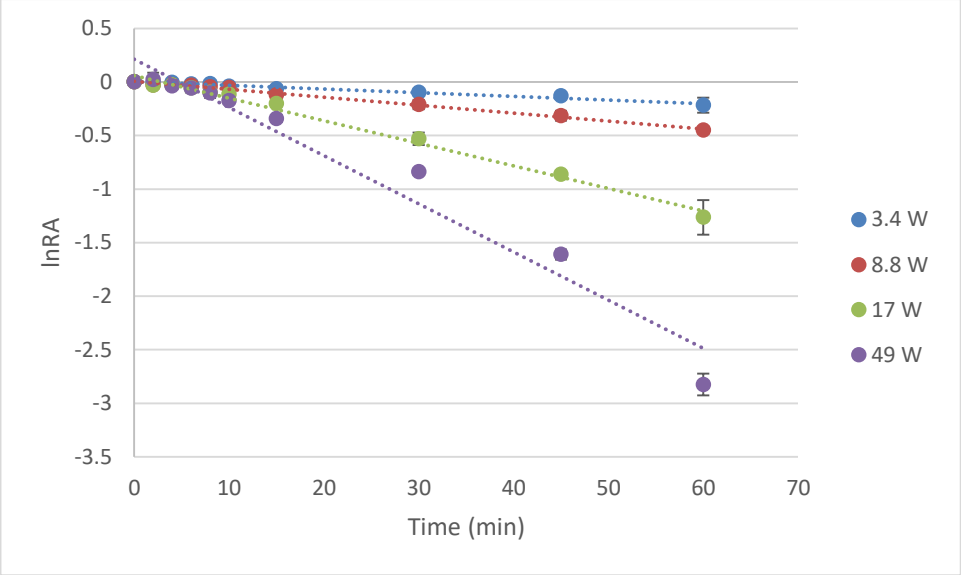


Figure 3.8 Inactivation kinetics curves of HRP at 1144 kHz and power levels ranging 3.4 – 49 W. Values presented are means (n=9) ± STDEV.

The inactivation kinetics of HRP after US treatment at 1175 kHz and 3.4, 15, and 39 W are shown in Fig. 3.9. Values of R^2 varied from 0.959 to 0.998, fitting well inactivation data into the first order kinetics model. Inactivation rate increased with increasing power, as indicated by k values varying from 0.8 to 7.4, and 20.9×10^{-3} min^{-1} for power applied at 3.4, 15, and 39 W. Additionally, sonication under these conditions had statistically significant effect on HRP activity ($p<0.05$), with RA % being recorded 94%, 59% and 27% after 60 min at 1175 kHz and 3.4, 15, and 39 W, respectively.

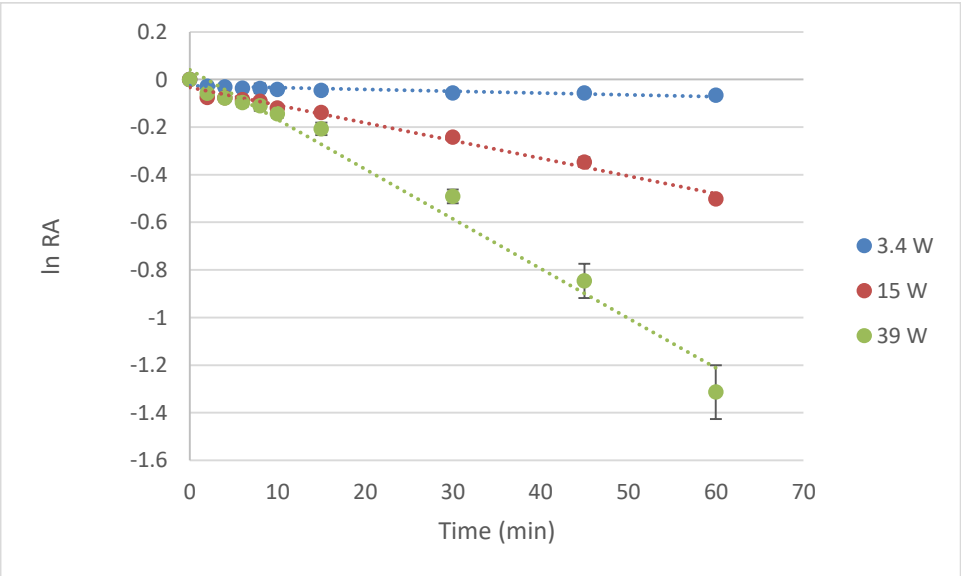


Figure 3.9 Inactivation kinetics curves of HRP at 1175 kHz and power levels ranging 3.4 – 39 W. Values presented are means ± STDEV, n=9.

Kinetics of peroxidase inactivation have been previously reported using various models. The most common is the first order kinetics model, according to which the behaviour of the enzyme inactivation mechanism is attributed to a single fraction of the enzyme. Biphasic inactivation model has also been used, which considers the existence of two fractions (labile and resistant) contributing to the total activity. Additionally, Weibull model has been employed in order to predict kinetics of POD inactivation, which assumes the presence of multiple isoenzymes catalysing the same reaction, but exhibiting different physicochemical and thermal resistance properties (Lindsay Rojas, Hellmeister Trevilin and Augusto, 2016).

First order inactivation kinetics of POD were observed by Cao *et al.* (2018), when they treated bayberry juice with US at 20 kHz and various amplitudes. Cruz, Vieira and Silva (2006) reported first order inactivation kinetics of watercress POD after thermosonication, whereas a biphasic behaviour was recorded after thermal treatment. The kinetics of the thermal treatment of POD fitted the biphasic model as observed by Lemos, Oliveira and Saraiva (2000). Thermosonication of POD resulted in first order inactivation kinetics, although there was no increase of the rate constant at higher power levels (De Gennaro *et al.*, 1999), which is not in accordance with the present study. On the other hand, Ercan and Soysal (2011) reported first order inactivation kinetics of tomato peroxidase after ultrasonic treatment at 23 kHz and elevated rate constants with increasing power, which is in good agreement with the findings of the current work within the range of all frequencies investigated. Recently, Rojas *et al.* (2017) examined the effect of US at 20 kHz on POD inactivation in coconut water, based on Weibull model.

As it can be deduced from the literature reports, there are various factors affecting enzyme inactivation and the respective kinetics. The external factors can be the temperature, time of process, type of treatment, while the source of the enzyme is also important.

The impact of different powers at the same frequency, as well as similar acoustic powers at different frequencies on the enzyme activity was

investigated during the current work. The overall results are shown in Table 3.2, together with the rate constant values derived from the kinetics analysis.

Low acoustic powers (<15 W), at low (20 kHz) or high frequencies (378, 583, 862, 995, 1144, 1175 kHz) resulted in statistically significant ($p<0.05$) decrease of HRP RA, however no efficient levels of inactivation regarding preservation purposes were achieved. In contrast, high frequencies and high acoustic powers decreased significantly ($p<0.05$) HRP activity, and complete inactivation was achieved when using 378 kHz and 583 kHz (at 48 W) and at 1144 kHz (49 W). The impact of similar acoustic powers (15 – 17 W) at low (20 kHz) and high frequency (378, 583, 995, 1114 and 1175 kHz) on HRP showed a variation. Similar residual enzyme activity was found when 1175 kHz at 15 W and 20 kHz at 16 W were used (59% and 62% respectively, $p<0.05$). On the other hand the application of 378, 583, 995 and 1144 kHz at 17 W, resulted in RA of 15, 34, 30 and 42%, respectively ($p<0.05$).

The use of 378 kHz at 32 W and 583 kHz at 34 W was found to be very effective on HRP inactivation, leading to 10% RA ($p<0.05$) after 45 min of sonication, while no RA was observed after 60 min of treatment. On the contrary, HRP RA was 28%, and 27% ($p<0.05$) when 20 kHz at 35 W and 1175 kHz at 39 W were applied for 60 min, respectively, whereas 995 kHz at lower power 25 W was more efficient resulting in 13% ($p<0.05$) HRP RA after 60 min of treatment.

Considering the frequencies and power levels studied, the most effective conditions for enzyme inactivation found to be either 378 or 583 kHz at 48 W. The application of either of these two frequencies at the highest power resulted

in the complete inactivation of the enzyme after 45 min of treatment, while 1144 kHz at 49 W resulted in 6% ($p<0.05$) HRP RA after 60 min of sonication.

The effect of heat at 40 ^{o}C on HRP activity was also examined for comparison purposes, given the fact that the maximum rise of temperature of the enzyme solution during sonication was 43 °C. After thermal treatment the enzyme exhibited a typical biphasic behaviour which is in accordance with previous work (Lemos, Oliveira and Saraiva, 2000). However, 60 min of treatment at 40 ^{o}C resulted in 79% ($p<0.05$) HRP RA, which suggests that HRP inactivation after high frequency sonication can possibly be attributed to US effect rather than that of heat.

Results of the statistical analysis are shown in Table 3.3 and indicate that there was an interaction between time*power ($F(1,2159)=644$; $p<0.001$), frequency*power ($F(1,330)=14.5$; $p<0.001$) and amongst time*frequency*power ($F(1,2158)=66.4$; $p<0.001$), however there was no significant interaction between time*frequency ($F(1,2158)=1.3$; $p=0.247$). The interaction amongst all three factors studied indicates that the rate of inactivation of HRP is not the same for the different treatments studied. There was also a main effect of time ($F(1,2160)=75.5$; $p<0.001$), frequency ($F(1,331)=7.6$; $p<0.05$), and power ($F(1,331)=25.3$, $p<0.001$). Nevertheless, the main effects of the individual factors are difficult to interpret in the presence of an interaction.

Table 3.2 Results of linear mixed model of ultrasonic treatments of HRP

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	336.576	3267.009	.000
Time	1	2159.855	75.555	.000
Frequency	1	331.142	7.643	.006
Power	1	331.645	25.380	.000
Time * Frequency	1	2158.358	1.339	.247
Time * Power	1	2158.669	644.416	.000
Frequency * Power	1	329.679	14.562	.000
Time * Frequency * Power	1	2158.006	66.433	.000

a. Dependent Variable: HPR_RA.

POD inactivation by low frequency/high power US has been previously reported in the literature. Cao *et al.* (2018) found a significant decrease of bayberry POD RA with increasing ultrasonic intensity and time. Complete inactivation of POD was achieved after US treatment at 452 W/cm^2 for 12 min. Sonication with cooling for 12 min at ultrasonic intensity of 90, 180, 271, 362 and 452 W/cm^2, resulted in 73%, 48%, 38%, 19%, and 9% POD RA respectively. They also observed that US treatment without cooling was more efficient in POD inactivation in bayberry juice, with RA being recorded as 58%, 20%, 8% and 1% at 90 – 362 W/cm^2 for 12 min. Ercan and Soysal (2011) reported that tomato POD RA was 64% and 16% after 150 s ultrasonic treatment at 23 kHz and powers of 25% and 40%, respectively, while complete inactivation was observed after 150 s and 90 s, at 50% and 75% power respectively. Saeeduddin *et al.* (2015) found that POD RA of pear juice was 43% after 10 min sonication (20 kHz, 750 W) at 45 ^{o}C, while 10 min sonication at 65 ^{o}C, completely inactivated the enzyme. The inactivation times mentioned in these studies are much shorter, as compared to the present work however, the US intensities/powers used were much higher, than in this study.

As it can be deduced from the results presented above, frequency, acoustic power and time of sonication affect enzyme activity; hence, it is possible to state that US causes alterations in the enzyme structure and subsequently its partial or total inactivation. Furthermore, increasing power at the same frequency leads to a decrease in the enzyme activity. However, the level of inactivation is very

much dependent upon the frequency used. On the other hand, it is not possible to conclude, that an increase of frequency at the same power can proportionally lead to a decrease of the enzyme activity. Delgado-Povedano and Luque de Castro (2015) in their review mention that a longer sonication time can cause an increase of bubble formation, while at higher powers there is a larger number of bubbles formed which after their collapse, create an adverse environment for the enzyme structure (high temperature and pressure).

Different mechanisms have been proposed for US induced enzyme inactivation, including cavitation and the formation of free radicals due to sonolysis of water (Kadkhodaee and Povey, 2008) which causes protein denaturation leading to enzyme inactivation (O'Donnell *et al.*, 2010). The free radicals generated by cavitation can also lead to alterations on the enzyme structure, as a consequence of the reaction between hydroxyl radicals and enzyme amino acids (Raviyan, Zhang and Feng, 2005; Terefe *et al.*, 2009). Additionally, US can also produce shock waves resulting in strong shear and microstreaming in the adjacent liquid. These extreme conditions caused by sonication, can lead to alterations in secondary and tertiary structures of enzyme and thence, into loss of biological activity (Cheng, Zhang and Adhikari, 2013). Lopez and Burgos (1995) attributed POD inactivation by manothermosonication to the removal of the haem group from enzyme's active site. An insight into a potential mechanism for HRP inactivation has been proposed in our previous work (Tsikrika *et al.*, 2017). Apart from the detachment of the haem moiety, which was observed after UV-visible spectroscopic analysis of US treated HRP solution; time resolved fluorescence spectroscopy analysis revealed the presence of a new fluorescent species in the solution. The new species was associated with the formation of di-tyrosine within the enzyme, while its origin has been attributed to the reaction of neighbouring tyrosine residues that was catalysed by the haem and hydrogen peroxide, produced by sonication of the water (see Section 5).

As aforementioned, there is limited information about the impact of power and ultrasonic frequency on food enzymes; however, there are a few studies on dosimeters and chemical pollutants, which give further insights. The effect of

US at 40 kHz and at different amplitudes on commercial cellulose was evaluated by Szabó and Csiszár (2013) and they observed that higher amplitude lead to higher degrees of enzyme inactivation. Kobayashi *et al.* (2012) reported higher degradation rates of methylene blue when higher ultrasonic power was applied, and they attributed this observation to enhanced cavitation and hence increased levels of hydroxyl radicals. It has been recorded that higher US frequencies generate more hydroxyl radicals than 20 kHz (Petrier *et al.*, 1992), while Comeskey *et al.* (2012) observed that the application of 850 kHz ultrasound was the most efficient in hydrogen peroxide production and dye decolourisation. Higher frequencies generate more cavitation bubbles than lower frequencies, although their implosion releases less energy due to their smaller size. However, it should be taken into account that the polarity of a particular substrate has a major impact on whether or not the species are able to get inside the cavitating bubble or reside on the exterior surface. Hydrophobic molecules can enter inside the cavitation bubble, whereas hydrophilic compounds stay at the bubble interface (Pétrier and Francony, 1997). The latter could be also the case of the present study, since the occurring reactions involved hydroxyl radicals.

To sum up, there is a complex interaction of factors, which designate the optimum frequency for the POD inactivation and comprise size and lifetime of the cavitating bubbles, geometry of the ultrasonic reactor, frequency, power, and amount of generated hydroxyl radicals; cavitation threshold; nature of the substrate, size and polarity of the enzyme (Bi *et al.*, 2015). In fact, according to Balachandran *et al.* (2016) there might be a unique frequency of maximum efficiency or reaction rate for every set of conditions.

3.4 Conclusion

Sonication at different frequencies and powers resulted in a decrease in HRP RA, while inactivation kinetics data fitted well in a first order model. Furthermore, inactivation rate increased with higher power at every applied frequency. Complete HRP inactivation was most efficiently achieved after 60 min of US treatment at either 378 kHz or 583 kHz and at a power level of around 48 W. Low frequency US at 20 kHz and 35 W lead to a 58% HRP RA

after 60 min of treatment, while 60 min of control (purely thermal) treatment at 40 ^{o}C only decreased HRP RA by 21%. The latter also suggests that HRP inactivation by high frequency sonication can be attributed to US effects rather than heat effects. Furthermore, the experimental findings indicate that there exists a frequency/amplitude combination to which corresponds to the maximum efficiency of the treatment. The mechanism of HRP inactivation probably involves detachment of the haem group due to loss of iron facilitated by hydroxyl radicals because of cavitation and further discussion can be found in Chapter 5.

4 Effect of Ultrasonic and Thermosonic Treatment on the Activity of Mushroom (*Agaricus bisporus*) Polyphenoloxidase

4.1 Introduction

Polyphenol oxidase (PPO; EC 1.14.18.1) is a copper containing enzyme commonly found in fresh fruit and vegetables and it is associated with the browning process (Cheng *et al.*, 2013). The browning reaction occurs from mechanical injury during post-harvest storage or processing of various fruit and vegetables (Fortea *et al.*, 2009). This reaction is catalysed by PPO and involves the oxidation of a monophenol and/or a o-diphenol into their corresponding o-quinone derivatives (Van Loey, Verachtert and Hendrickx, 2001; Ismaya *et al.*, 2011). In the presence of oxygen the ortho-quinones undergo further polymerisation to yield undesirable brown pigments (Jang and Moon, 2011). A simplified version of the reaction can be seen below in fig 4.1.

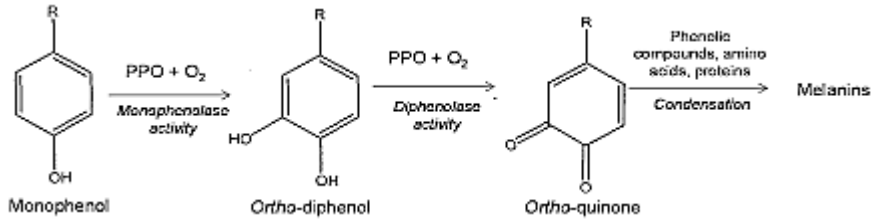


Figure 4.1 Simplified version of PPO catalysed browning reaction (Taranto et al.*, 2017)*

Enzymatic browning is associated with off-flavours and ultimately contributes significantly to quality loss of the nutritional and market value of food and food products. Therefore, the inhibition of enzymatic browning is a requirement in the food industry (Cheng, Zhang and Adhikari, 2013).

Thermal processes are the most common and widely employed techniques for enzyme inactivation in food industry (Gouzi, Depagne and Coradin, 2012). However heat treatments might result in the development of undesirable flavours and deterioration of functional properties of food products as well as nutrient loss (Chemat *et al.*, 2011). Hence, ultrasound, an environmentally friendly technique with relatively low chemical and physical hazards has been investigated in the last two decades as full or partial alternatives to conventional heat treatment (Chemat, Zill-e-Huma and Khan, 2011; Costa *et al.*, 2013).

The application of ultrasound, alone or in combination with other methods, regarding PPO inactivation, is increasingly studied. However, most of studies performed are using low frequency. Recently, Cao *et al.* (2018) reported significant decrease in PPO activity in bayberry juice after sonication at 20 kHz US, same as Saeeduddin *et al.* (2015) observed in pear juice. Another recent study by Liu *et al.* (2017) examined the effect of low frequency/ high power US (20 kHz, 100 – 500 W) on PPO in oriental sweet melon. PPO inactivation was only observed when power above 200 W was used. PPO inactivation by high intensity US at 19 kHz was also observed by Costa *et al.* (2013) in pineapple, and by Fonteles *et al.* (2012) in cantaloupe melon juice. On the contrary, Bi et al. (2015) found that US at 20 kHz increased PPO activity in avocado puree.

The use of US in conjunction with other methods has also been studied, in order to achieve faster PPO inactivation. Significant PPO inactivation in mushroom by thermosonication has been reported by Cheng, Zhang and Adhikari (2013), and Baltacıoğlu, Bayındırlı and Severcan (2017) and in pear, apple and strawberry purees by Sulaiman *et al.* (2015), and the application of manothermosonication significantly decreased PPO activity in a model system (Lopez *et al.*, 1994). Furthermore a combined treatment of ultrasound and high hydrostatic pressure significantly decreased PPO activity in apple juice (Abid *et al.*, 2014; Bot *et al.*, 2018), and Başlar and Ertugay (2013) reported significant PPO inactivation in apple juice by photosonication. Additionally, a simultaneous treatment of ultrasound and ascorbic acid resulted in decrease in PPO activity in fresh-cut apple (Jang and Moon, 2011).

Nevertheless, there is no available information on the effect of higher US frequencies on PPO activity. Previous work on model system of HRP (Tsikrika *et al.*, 2018) showed that high rather than low frequency is more efficient in enzyme inactivation. Hence, the aim of this study is twofold; (i) to evaluate the effect of US on PPO activity and define the respective inactivation kinetics, (ii) to investigate the effect of ultrasound on previously thermally treated PPO samples.

Specifically, the objectives of this work are:

- To examine the effect of low and high US frequency (20, 378, 583, 1144, and 1175 kHz) on PPO activity at selected different acoustic powers (7 – 49 W) and times in order to gain a better understanding of the of US technology for enzyme inactivation.
- To determine the inactivation kinetics of PPO upon the US treatments
- To compare the US induced PPO inactivation with purely thermal treatments at 40 ^{o}C, 50 ^{o}C, and 60 ^{o}C.
- To investigate the effect of thermal pre-treatment at 40 ^{o}C, 50 ^{o}C, and 60 ^{o}C, followed by sonication at the same temperature on PPO activity, in order to minimise the inactivation time.

4.2 Materials and methods

4.2.1 **Chemicals**

Catechol, sodium phosphate monobasic, sodium phosphate dibasic, polyvinylpolypyrrolidone (PVPP), triton X-100 were purchased from Sigma-Aldrich, Gillingham, UK. All chemicals were of analytical grade.

4.2.2 **Raw materials**

Freshly harvested chestnut mushrooms (*A. bisporus*) were purchased from the local market in Dundee, UK. The mushrooms were selected, washed thoroughly and then processed in order to produce a crude PPO extract.

4.2.3 **Preparation of crude PPO extract**

A modified method of Sulaiman *et al.* (2015) was followed in order to prepare the crude enzyme extract. Mushrooms (500 g) were cut into thin slices and suspended in sodium phosphate buffer solution (1 L, 0.1 M; pH 6.0) containing 4% (w/v) insoluble PVPP with the addition of 1% (v/v) triton X-100 and mixed for 3 min in a kitchen blender. The homogenate was then centrifuged at 10,000 g for 30 min at 4 ^{o}C and the supernatant was collected. This liquid constituted the crude PPO extract for this study and was kept frozen at -15 ^{o}C until use. When required, an aliquot of the frozen extract was thawed at room temperature and diluted 1:1 (v/v) with sodium phosphate buffer.

4.2.4 **Ultrasonic treatment**

The ultrasound equipment used in these experiments was a Misonix Ultrasonic Liquid Processor operating at 20 kHz or a Meinhardt Ultraschlltechnik high frequency sonicator (HM8001-2, Leipzig, Germany) as described in Section 3.2.3.

4.2.4.1 Use of 20 kHz sonicator

A standard volume of diluted extract (200 mL) was put into a 400 mL beaker which was then placed in a 2 L ice bath filled with crushed ice and tap water (400 mL). The 20 kHz sonicator probe was positioned, consistently, 20 mm from the bottom in the 400 mL beaker. A thermometer was also suspended in the beaker to allow the temperature to be monitored. The 20 kHz sonicator experiments were all conducted at 35 W and operated on a pulse mode of 4 s on and 2 s off until 90 min of sonication were completed. Samples were removed after 10, 20, 30, 40, 50, 60, 75 and 90 min of sonication and kept in ice until they were analysed.

4.2.4.2 High frequency sonication

A standard volume of diluted extract (200 mL) was put in the reaction vessel, a thermometer was suspended in the reaction liquid and cooling was achieved with a flow of cold water through the reactor jacket. The high frequency sonicator amplitude was set at appropriate amplitude, depending on the specific frequency chosen, and sonicated for 90 min. Samples were withdrawn after 0, 10, 20, 30, 40, 50, 60, 75 and 90 min of sonication and kept in ice until they were analysed.

4.2.5 **Thermal treatment**

A 250 mL Duran glass bottle containing 200 mL of diluted extract was placed in the middle of a water bath previously equilibrated at 40^{o}C, 50 ^{o}C, and 60^{o}C. At predetermined time intervals (10, 20, 30, 40, 50, 60, 75 and 90 min) samples would be removed of the bath and then they would immediately be immersed in an ice bath and kept there prior to analysis.

4.2.6 **Thermosonic treatment**

A 250 mL Duran glass bottle containing 200 mL of diluted extract was placed in the middle of a water bath at 40^{o}C, 50^{o}C, and 60^{o}C. A thermometer was also suspended inside the glass bottle in order to allow the temperature to be monitored. When the specified temperature was reached, the extract was transferred to the jacketed glass reaction vessel connected to the high frequency sonicator and sonicated at 378 kHz and 48 W. The temperature was maintained at specified values (40, 50, and 60 ^{o}C (±2 ^{o}C), respectively) with a water flow through the reactor jacket. Samples were removed after 10, 20, 30, 40, 50, 60 and 90 min of sonication, immediately immersed in an ice bath and kept there until they were analysed.

4.2.7 **PPO activity assay**

PPO activity (ΔA_{420} min^{-1} g^{-1} FW) was assayed according to a modified method of Buckow *et al.* (2009). Diluted extract (150 µL) was mixed with catechol solution (0.5 mL; 0.05 M in 0.1 M sodium phosphate buffer, pH 6.0) and sodium phosphate buffer (1.5 mL; 0.1 M pH 6.0). Oxidation of catechol was then measured on a UV spectrophotometer at 420 nm and 25 °C every 10 s for a total duration of 150 s (UV-1650 PC, Shimadzu UK Ltd). The residual activity (RA) of PPO was calculated using the following equation:

$$\text{Residual Activity } (\%) = \frac{\mathrm{A_t}}{A_0} \times 100$$

Equation 4.1

where A_t and A_0 are, respectively, PPO activity after and before the treatment.

4.2.8 **Statistical analysis**

Statistical analysis was performed using Mixed-Linear model by IBM SPSS Statistics 23. A mixed effect model was produced with time (min), frequency (kHz) and power (W) as covariates to examine any possible interaction between power, frequency and time at each experiment. Variation between replicate samples was treated as a random factor. Linearity between the predicted values and the dependent variable (PPO RA), normality of distribution of residuals, and independence of residuals were also checked (the plots can be found in the

Appendix A, Fig A 6 – A 8). Values presented are the mean of experiments done in triplicate and replicated 3 times (n = 9). The values were considered significantly different when $p<0.05$.

4.2.9 **Kinetics of PPO inactivation**

The inactivation kinetics of mushroom PPO were evaluated based on the Weibull model, which considers the biological variation in order to describe the range of responses of a system to an adverse factor under different conditions (Peleg and Cole, 1998). According to Weibull model the inactivation mechanism may differ amongst macromolecules, hence the survival curve could be represented as the cumulative form of the underlying distribution of the different inactivation times (Jaramillo Sánchez *et al.*, 2018). The Weibull model can be described by the equation 4.2 below:

$$A_t = A_0 e^{-(\frac{t}{\alpha})^{\gamma}}$$

Equation 4.2

where, A_t is the activity at time t, A_0 is the initial activity, t is the treatment time (min), α is the scale factor (min) and γ is the shape parameter, which indicates the concavity or convexity of the curve (Pankaj, Misra and Cullen, 2013; Quintão-Teixeira *et al.*, 2013).The calculations and the plots for the Weibull model were performed using Origin software.

4.3 Results and Discussion

Different frequencies (20, 40, 378, 583, 1144 and 1175 kHz) and acoustic powers (7 – 49 W) were used to examine their influence on PPO activity and define the respective inactivation kinetics. All experiments resulted in significant ($p<0.05$) decrease in enzyme activity, while the maximum temperature reached during sonication experiments was 43 ^{o}C. It should also be pointed out that enzyme reactivation did not occur after any treatment.

PPO inactivation kinetics regarding food processing has been described by various models. The most common is the first order kinetics model (Cheng, Zhang and Adhikari, 2013; Sulaiman *et al.*, 2015), however biphasic behaviour has also been reported (Ludikhuyze *et al.*, 2003). Nevertheless, plants naturally

contain isozymes i.e. several forms of the same type of enzyme which catalyse the same reaction, but may exhibit different physicochemical and thermal resistance properties (Shannon, Kay and Lew, 1966). Hence, in order to represent these complex mechanisms of enzyme inactivation, the Weibull model has also been employed as more realistic and flexible (Rojas *et al.*, 2017).The Weibull model has been used to describe enzyme inactivation by various non-thermal processes such as ultrasound (Rojas *et al.*, 2017), pulsed electric fields (Giner *et al.*, 2005; Elez-Martínez, Aguiló-Aguayo and Martín-Belloso, 2006; Soliva-Fortuny, Bendicho-Porta and Martín-Belloso, 2006; Quintão-Teixeira *et al.*, 2013), high hydrostatic pressure (Buckow, Weiss and Knorr, 2009), atmospheric pressure cold plasma (Pankaj, Misra and Cullen, 2013), and ozonation (Jaramillo Sánchez *et al.*, 2018). First order and biphasic model were tested with the data of this study, but very poor fits were obtained. However, Weibull model fitted well the data and the overall results for the different US treatments of mushroom PPO examined in the present work are shown in Table 4.1.

Table 4.1 Rate constants (min^{-1}), shape parameter values (±STDEV), and adjusted correlation coefficients (R^2) for different US treatments of mushroom PPO

Frequency	Power	Rate Constant ($x10^{-3}min^{-1}$)	Shape Parameter	Adjusted R^2
20	35	5.4 ± 1.1	0.9 ± 0.2	0.980
378	10	9.5 ± 5.2	1.8 ± 0.19	0.997
	32	22 ± 0.8	1.8 ± 0.06	0.999
	48	23 ± 0.7	2.4 ± 0.16	0.997
583	34	24 ± 2.3	1.8 ± 0.02	0.999
	48	28 ± 3.6	1.7 ± 0.05	0.999
1144	49	23 ± 2.4	1.9 ± 0.04	0.999
1175	39	21 ± 0.3	1.4 ± 0.2	0.999

The inactivation kinetics curves of PPO when a frequency of 20 kHz was applied at 35 W can be seen in the figure below (Fig 4.2). The value of the shape parameter found to be 0.9, and of the adjusted correlation coefficient $\left(R^2_{adj}\right)$ 0.980, while inactivation rate constant (k-value) was 5.4×10^{-3} min^{-1}. The fact that the shape parameter value after US treatment at 20 kHz and 35 W is approximately 1 indicates that the enzyme population has a higher homogeneity

i.e., more isozymes with similar heat stability, and subsequently with similar critical time of inactivation (Peleg and Cole, 1998). Therefore, these PPO inactivation kinetics data designate the presence of a first order model, which is also easily deduced from the graph, since the shape of the “curve” is close to a linear form

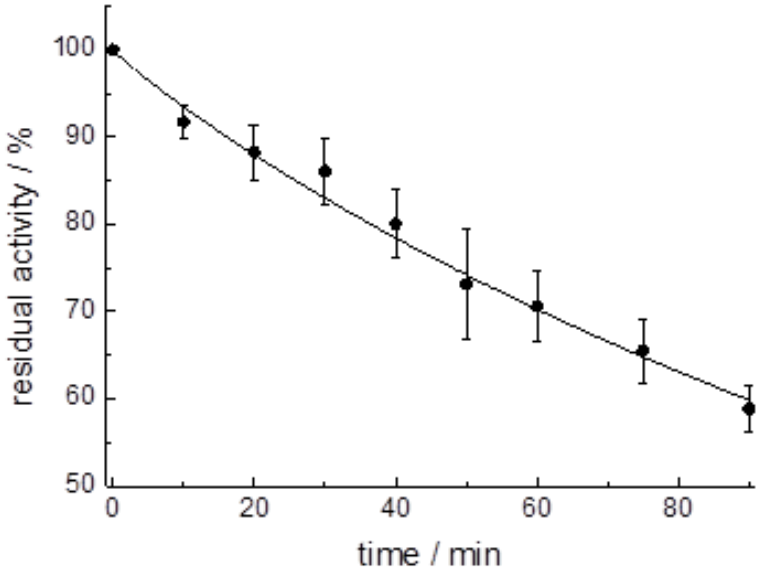
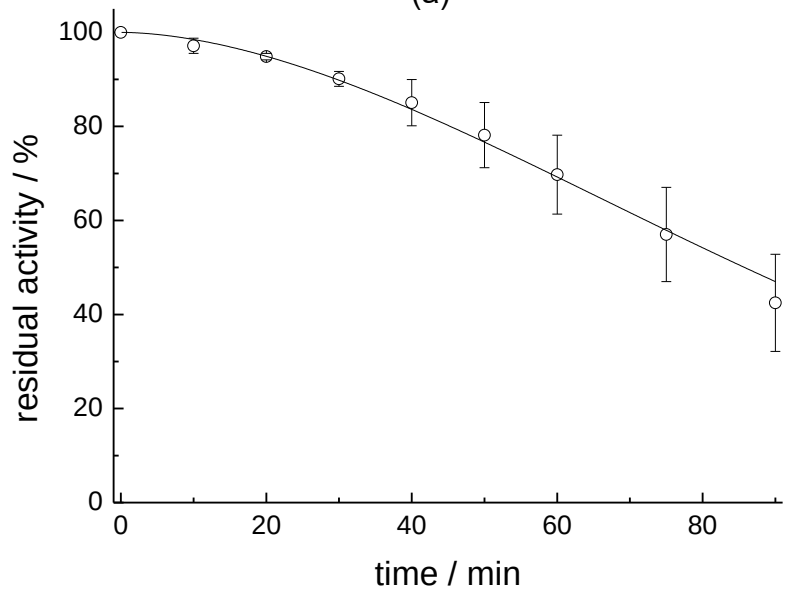


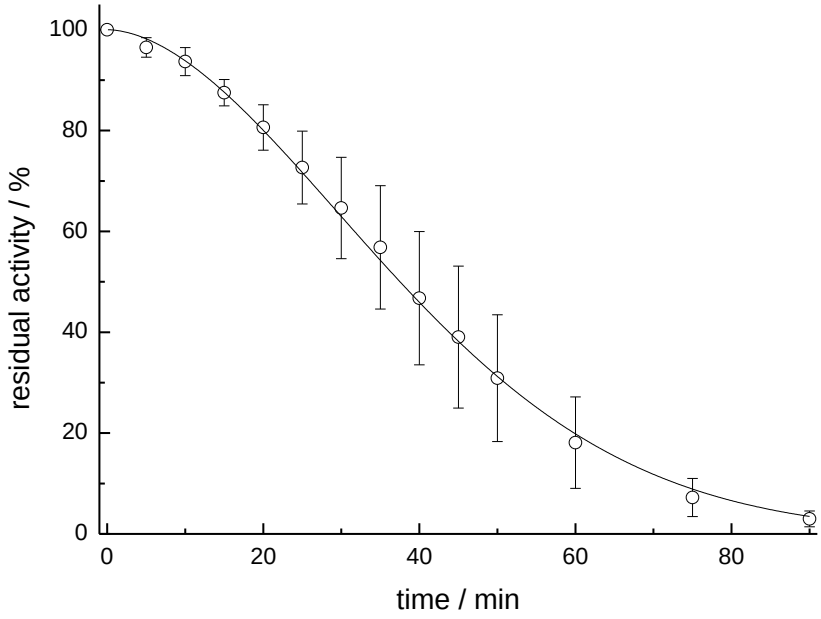
Figure 4.2 Inactivation curve of PPO after sonication at 20 kHz and 35 W. Values presented are the average (n=9) ± STDEV.

The effect of high frequency US at 378 kHz and 10, 32, and 48 W on PPO inactivation kinetics can be seen on the figures below (Fig 4.3 a, b, and c). The shape parameter varied from 1.8 to 2.4, and the R^2_{adj} values of 0.997 to 0.999. Inactivation rate increased with increasing ultrasonic power with the k-values being recorded as 9.5×10^{-3} min^{-1}, 21.7×10^{-3} min^{-1}, and 23×10^{-3} min^{-1}, after sonication at 378 kHz and 10, 32, and 48 W, respectively.

(a)


residual activity / %
time / min
0
20
40
60
80
100

(b)


residual activity / %
time / min
0
20
40
60
80
100

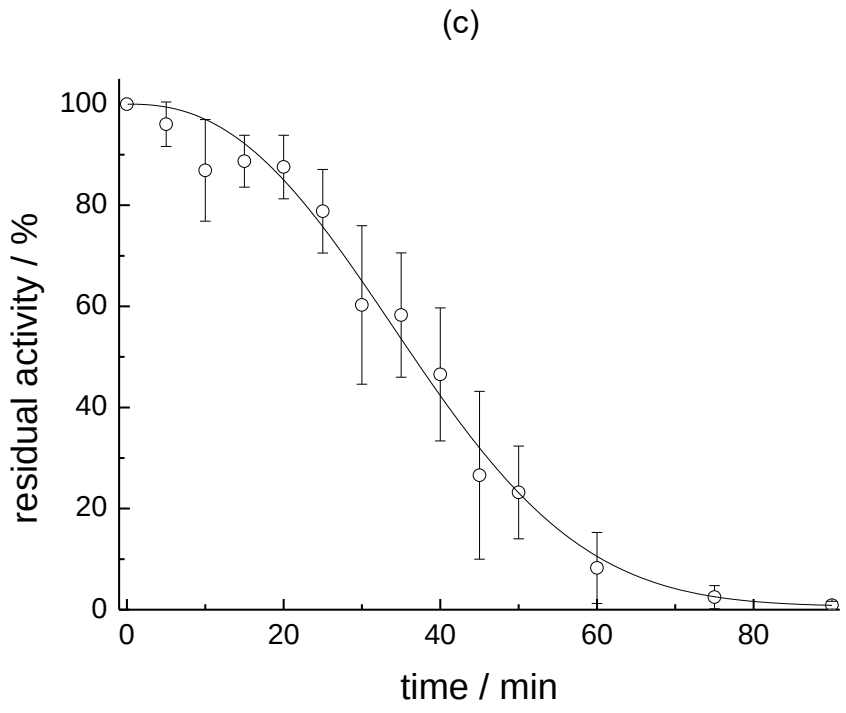


Figure 4.3 Inactivation curve of PPO after sonication at 378 kHz and 10 W (a), 32 (b), and 48 W (c). Values presented are the average (n=9) ± STDEV.

As shown below (Fig 4.4 a and b) inactivation kinetics of PPO by US at 583 kHz and power level of 34, and 48 W were predicted well by Weibull model with the shape parameter values ranging from 1.7 to 1.8, and R^2_{adj} values of 0.999, in both cases. K-values were recorded as 24×10^{-3} min^{-1}, and 28×10^{-3} min^{-1}, for sonication at 583 kHz and power of 34, and 48 W, respectively, indicating a slight increase in inactivation rate, with higher power.

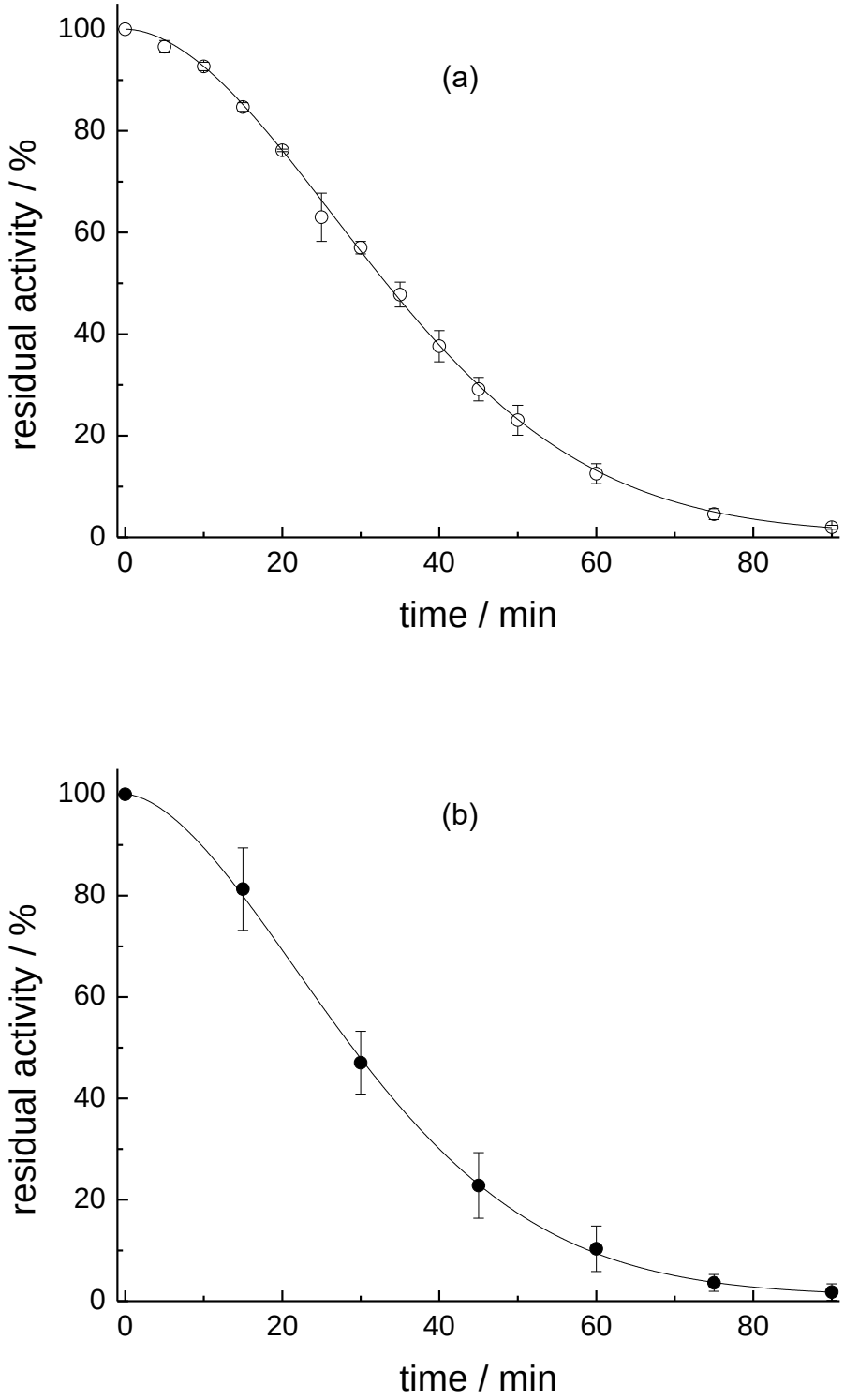


Figure 4.4 Inactivation curve of PPO after sonication at 583 kHz and 34 W (a), and 48 W (b). Values presented are the average (n=9) ± STDEV.

Figure 4.5 shows the effect of 1144 kHz and 49 W on the inactivation of PPO. The shape parameter found to be 1.9 and R^2_{adj} 0.999, while k-value was recorded as 23×10^{-3} min^{-1}.

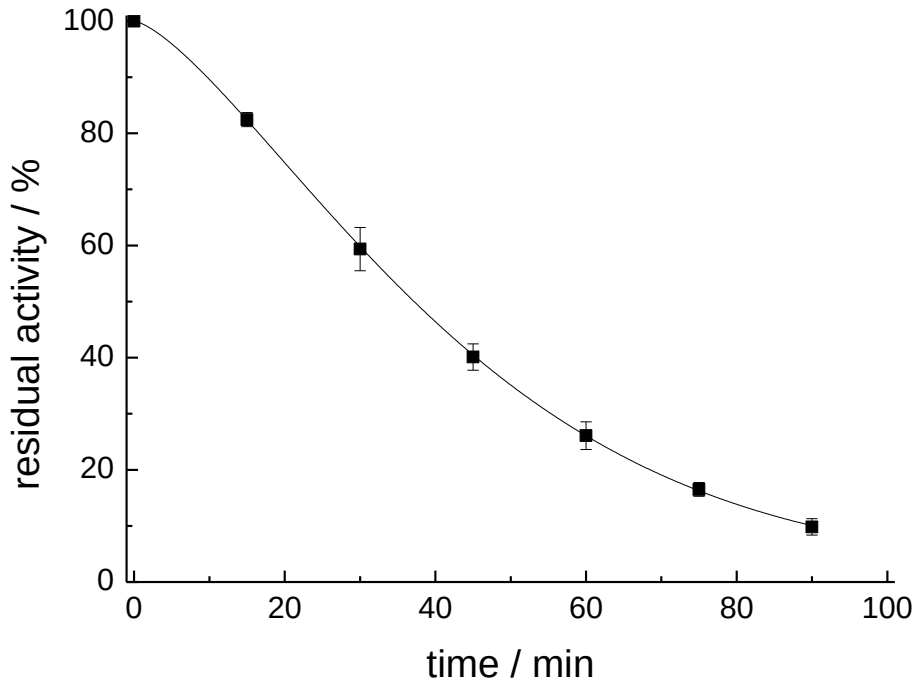


Figure 4.5 Inactivation curve of PPO after sonication at 1144 kHz and 49 W. Values presented are the average (n=9) ± STDEV.

The inactivation curve of PPO upon US treatment at 1175 kHz and 39 W is shown in Fig 4.6. The shape parameter was recorded as 1.4, the R^2_{adj} value 0.999, and a k-value of 21×10^{-3} min^{-1}.

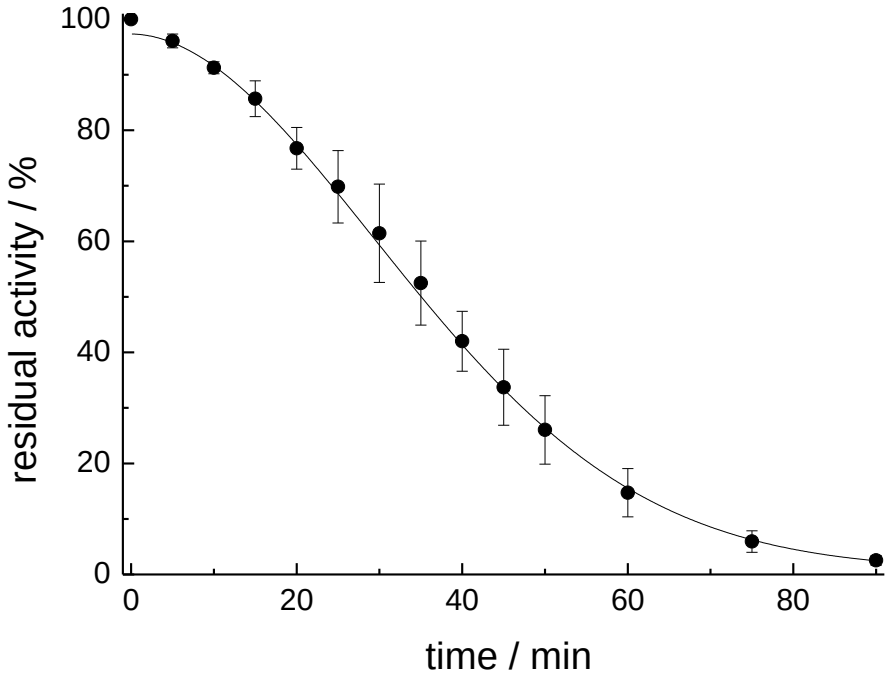


Figure 4.6 Inactivation curve of PPO after sonication at 1175 kHz and 39 W. Values presented are the average (n=9) ± STDEV.

The effect of the low frequency at 20 kHz and high frequencies 378, 583, and 1175 kHz at similar power levels 35, 32, 34, and 39 W, respectively was also investigated. The application of high frequency ultrasound at 378 kHz (32 W), 583 kHz (34 W), and 1175 kHz (39 W) resulted in a similar RAs of 3%, 2%, and 2.5%, respectively after 90 min of treatment, whereas the application of 20 kHz (35 W), for the same period, proved to be less effective at 59% RA.

The influence of low frequency (19 – 25 kHz) ultrasonic treatment on PPO activity has been reported, as aforementioned, however studies have shown a wide variation. Cao *et al.* (2018) observed 53%, and 13%, decrease in PPO RA of bayberry juice after 10 min sonication without cooling at 20 kHz and intensities of 90 and 181 W/cm^2 respectively, while at the higher intensities of 271 and 362 W/cm^2, PPO RA decreased 96% and 99% after 8 min, respectively. However, when the same conditions were applied with cooling (using an ice bath) inactivation was achieved relatively slowly. Saeeduddin *et al.* (2015) treated pear juice with ultrasound at 20 kHz using a 750 W probe and 70% amplitude for 10 min at 25 ^{o}C, 45 ^{o}C, and 65 ^{o}C and they found that PPO RA was 89%, 38%, and 2%, respectively. The inactivation times of these

studies are shorter than of those found in the current work. However, the intensities that they used were much higher than of those of the present study.

Costa *et al*. (2011) treated pineapple juice at 19 kHz and power levels of 100, 300, & 500 W for 2-10 min and they observed that PPO activity increased when intensity levels from 150 to 300 (W/cm^2), were applied, whereas decreasing trends were recorded at 500 W for processing times above 5 min. Dias *et al.* (2014) applied same frequency of 19 kHz, at intensities of 75,118, 224, 330 and 373 W/cm^2 for 2-10 min to soursop juice and noted only a slight decrease in PPO RA, irrespective of the processing time and power used. On the other hand, Abid *et al.* (2014) treated apple juice with ultrasound at 25 kHz, and 70 % amplitude for 60 min and did not notice any significant decrease in PPO RA, while Yu *et al.* (2013) reported an increase in PPO activity when treating the enzyme using an ultrasonic bath operating at 40 kHz and 100 W. They attributed these results to various factors such as mass transfer due to micro-mixing, enzyme release because of cell break-up and stimulation of biochemical reactions within cell tissues resulting in enhancement of the production of specific enzymes. Similar results were observed by Bi *et al.* (2015), who reported that the use of ultrasound at 20 kHz and 50% power (full power 750 W/cm^2), for 1-10 min resulted in increase in the RA of PPO extracted from avocado puree. However, such enzymatic behaviour was not observed under the test conditions of the current work. These variations in reported PPO inactivation kinetics could be possibly attributed to the different enzyme extraction sources, differences between crude and purified enzyme, as well as in difference in enzyme response towards the various conditions studied (Cheng, Zhang and Adhikari, 2013; Rojas *et al.*, 2017).

In the present study, low frequency US had a limited effect on enzyme activity, whereas the application of higher frequencies was more effective. The most effective conditions for PPO inactivation, regarding ultrasonic treatment were 378 kHz at 48 W, resulting in less than 1% RA after 90 min of treatment. The same acoustic power (48 W) at 583 kHz had a similar effect on the enzymatic activity leading to less than 2% RA while similar power level (49 W) at a higher frequency of 1144 kHz resulted in 10% RA.

The results of the statistical analysis are shown in Table 4.2. There was a significant interaction between time*frequency ($F(1,694)=208$; $p<0.001$), time*power ($F(1,695)=245$; $p<0.001$), and amongst time*frequency*power ($F(1,694)=181$; $p<0.001$), however there was no significant interaction between frequency*power ($F(1,2158)=1.3$; $p=0.247$). The interaction amongst all three factors studied indicates that the rate of inactivation of PPO is not the same for the different treatments studied.

There was also a significant effect of time ($F(1,2160)=75.5$; $p<0.001$), which is difficult to interpret because of the presence of the interaction, whereas frequency and power did not have a significant effect ($p>0.05$).

Table 4.2 Results of linear mixed model of ultrasonic treatments of PPO

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	126.485	119.043	.000
Time	1	694.431	62.949	.000
Frequency	1	124.607	.206	.651
Power	1	127.580	.315	.576
Time * Frequency	1	694.134	207.951	.000
Time * Power	1	694.860	245.443	.000
Frequency * Power	1	129.491	.336	.563
Time * Frequency * Power	1	694.262	181.094	.000

a. Dependent Variable: PPO_RA.

The effect of heat alone at 40 ^{o}C, 50 ^{o}C, and 60 ^{o}C (± 3 ^{o}C) on the activity of PPO was also examined. A slight decrease in PPO RA was observed over the 90 min of thermal treatment at 40 ^{o}C and the RA of the enzyme was 87%. Heating at 50 ^{o}C for 90 min resulted in a RA of 21%, whereas PPO lost more than 99% of its activity after 50 min at 60 ^{o}C ($p<0.05$). These results are in good agreement with Cheng, Zhang and Adhikari (2013) who reported 54% and 9% PPO RA after 30 min of heating at 55 ^{o}C and 60 ^{o}C, respectively.

The statistical analysis in Table 4.3 shows that there is a significant interaction between temperature*time ($F(1,237)=42$; $p<0.001$), indicating that the rate of inactivation of PPO is not the same for the different temperatures studied.

Temperature (F(1,237)=94; p,0.001) and time (F(1,237)=293; p<0.001) have a significant main effect as well but is difficult to interpret because of the presence of the interaction.

Table 4.3 Results of linear mixed model of thermal treatments of PPO

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	237	2375.386	.000
Temperature	2	237	94.176	.000
Time	1	237	292.847	.000
Temperature * Time	2	237	41.989	.000

a. Dependent Variable: PPO_RA.

Even though complete inactivation of PPO was achieved with US treatment alone, the duration of the process would be prohibitive for an industrial application. Therefore, the combined effect of heat and US on PPO activity was also studied at 378 kHz, which found to be the most effective regarding PPO inactivation. Fig 4.7 shows the inactivation kinetics during thermosonication treatments at 378 kHz and the aforementioned temperatures.

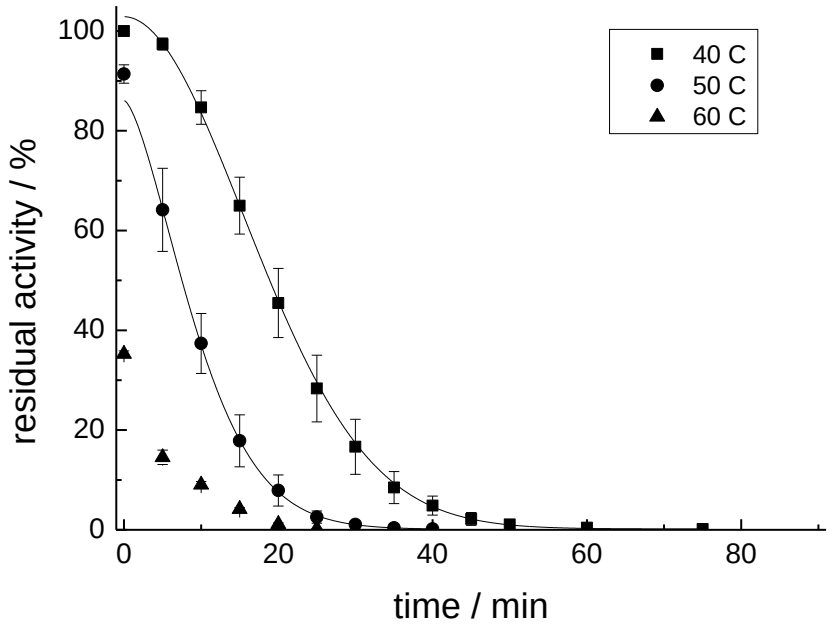


Figure 4.7 Inactivation curves of PPO after pre-treatment at 40, 50 and 60 ^{o}C followed by US at 378 kHz and 48 W.

Pre-treatment of PPO at 40 ^{o}C for 5 min, followed by US at 378 kHz and 48 W, (at 40 ± 3 ^{o}C) inactivated the enzyme after 50 min. Fitting the data to the Weibull model gave a shape parameter of 1.9 ± 0.04, while R^2_{adj} was 0.999. Heating at 50 ^{o}C, for 5 min followed by application of 378 kHz and 48 W (at 50 ± 3 ^{o}C) resulted in 1% RA after 30 min of US treatment. Pre-treatment at 60 ^{o}C for 5 min followed by ultrasonic treatment at 378 kHz at 48 W (at 60 ± 3 ^{o}C) inactivated PPO (RA <1%) after 20 min of sonication suggesting that there is a combined effect of heat and ultrasound, since thermal treatment at 60 ^{o}C for 20 min resulted in 12% RA, while 20-min long sonication at 378 kHz and 48 W lead to 88% RA. However, inactivation kinetics data did not fit the Weibull model.

A synergistic effect of heat and sonication on PPO inactivation has also been observed by Cheng, Zhang and Adhikari (2013), who studied the effect of thermal and thermosonic treatments on PPO in mushroom at 25 kHz (50% power) and 55 – 75 °C. They reported that thermosonication at 60 ^{o}C was more effective in PPO inactivation, than thermal treatment at 60 ^{o}C or sonication at 45 ^{o}C. PPO RA found to be ~ 1.0% after a 15-min long thermosonication treatment at 60 ^{0}C, while 3 min of treatment at 75 ^{0}C completely inactivated the enzyme. Sulaiman *et al.* (2015) observed no significant effect of thermosonication treatment at 24 kHz, 32.5 W and at temperature 32 and 52 ºC on PPO activity in pear, apple and strawberry. However at 72 ^{o}C PPO RA was significantly lower in all three fruits. Baltacıoğlu *et al.* (2017) observed that mushroom PPO RA was 61% and 12% at 40 and 50 ^{o}C, respectively after thermosonication treatment at 60% amplitude (24 kHz, 400 W) for 30 min. They also reported 99% PPO RA after 15 min at 80% amplitude or 20 min at 60% amplitude thermosonication at 60 ^{o}C which is in accordance with the findings of the present work.

Results of the statistical analysis can be seen in Table 4.4. There is a significant interaction between temperature*time*frequency ($F(1,477)=21$; $p<0.001$) and between temperature*time ($F(1,477)=21$; $p<0.001$), indicating that the rate of inactivation of PPO is not the same for the different frequencies and temperatures studied. Temperature ($F(1,237)=94$; $p=0.016$) and time ($F(1,237)=293$; $p<0.001$), has also a significant main effect, but is difficult to

interpret because of the presence of the interaction. On the other hand, frequency does not have a significant main effect (F(1,477)=0.7; p>0.05).

Table 4.4 Results of linear mixed model of thermosonic treatments of PPO

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	477.000	105.046	.000
Temperature	2	477	4.147	.016
Time	1	477	13.305	.000
Frequency	1	477.000	.675	.412
Temperature * Time	2	477	21.082	.000
Temperature * Frequency	2	477	1.933	.146
Time * Frequency	1	477	2.696	.101
Temperature * Time * Frequency	2	477	20.973	.000

a. Dependent Variable: PPO_RA.

The inactivation of enzymes by ultrasound is mainly associated with the mechanical and chemical processes that occur as a consequence of cavitation (Raviyan *et al.*, 2005). Consequently, the secondary and tertiary structures of enzyme are altered resulting into loss in their biological activity (Tian *et al.*, 2004). Liu *et al.* (2017) reported denaturation of purified PPO due to disruption of the *α*-helix structure after low frequency/ high power US treatment, as determined by circular dichroism spectroscopy analysis. They also observed alterations in the tertiary structure of sonicated samples of purified PPO, with the fluorophores becoming more buried and encapsulated in the protein, as indicated by fluorescence spectroscopy analysis. Baltacıoğlu *et al.* (2017) suggested that thermosonication inactivation of mushroom PPO was not due to an insignificant change in the active site, but because of a global conformation change of the enzyme. Another possibility is that the sonolysis of water generate hydroxyl radicals, that react with enzyme amino acids, which are then no longer able to participate in enzyme stabilisation, substrate binding or catalytic function which leads to enzyme inactivation (Barteri *et al.*, 2004). The inactivation of mushroom PPO by ultrasound, as presented in this study, might have been caused by one or a combination of those mechanisms. Further research is required to elucidate the specific mechanism(s) by which the

mushroom PPO might be inactivated. An insight into a possible mechanism of PPO inactivation upon US is discussed in the following section.

4.4 Conclusion

All US treatments caused a statistically significant ($p<0.05$) decrease in mushroom PPO RA. PPO inactivation kinetics data after low frequency US treatment at 20 kHz and 35 W followed a first-order model. The data obtained from PPO inactivation of sonicated samples at higher frequencies (378 – 1175 kHz) fitted the Weibull model well. Additionally, complete PPO inactivation was achieved after the application of 378 kHz at a power level of 48 W after 90 min, while sonication at 20 kHz and 35 W resulted in 59% RA after 90 min of treatment. Control thermal treatment at 40 ^{o}C for 90 min decreased PPO RA only by 13%, indicating that PPO inactivation by US is attributed to a sonic rather than heat effect. The most effective treatment regarding mushroom PPO inactivation was thermosonication at 60 ^{o}C, and 378 kHz (48 W) for 20 min, indicating a combined effect of heat and ultrasound on PPO inactivation. More research is needed to clarify possible mechanism(s) of PPO inactivation upon US treatment and is further discussed in the next section.

5 Time-resolved fluorescence observation of horseradish peroxidase and mushroom tyrosinase upon ultrasound treatment

5.1 Introduction

Fluorescence is a phenomenon caused by the interaction of light with matter which is then followed by the emission of light at a different wavelength. The timescale for this emission after a photon has been absorbed by a molecule (fluorophore) is on the pico- to nanosecond timescale (Valeur, 2001). Excited molecules can lose energy to their environment leading to an emission at a longer wavelength because of their lower energy. Fluorescence concerns the electronic transitions between singlet energy levels (Lakowicz, 2006). Although it is possible to excite molecules using chemical or electrical energy (chemo- and electroluminescence) typically light is used to provide energy to promote an electron from one molecular orbital to another.

Fluorescence can be represented by a Jablonski diagram, an adaptation of which can be seen in Fig 5.1. The singlet ground and first electronic state are depicted by S_0, and S_1, respectively, while the transitions between states are depicted as vertical lines. Fig 5.1 also indicates the transitions for the absorption (a), fluorescence (f), and phosphorescence (p) processes along with their relative spectral positions.

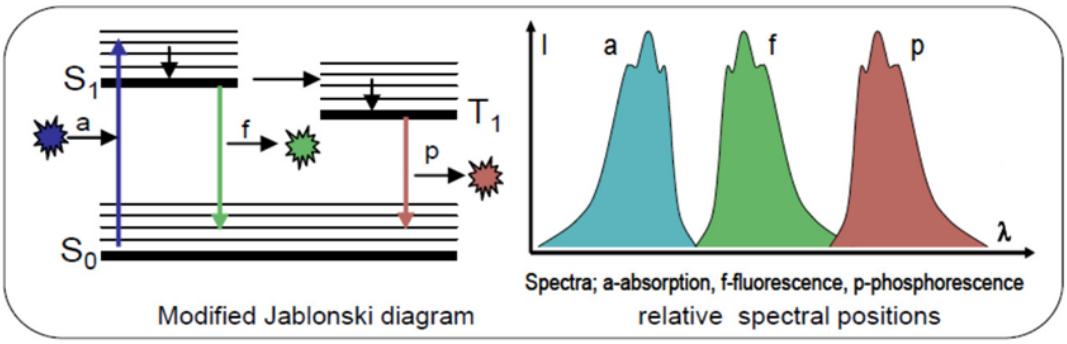


Figure 5.1 Graphical depiction of a modified Jablonski diagram (on the left) and of the relative wavelength positions of absorption (a), fluorescence (f) and phosphorescence spectra (p) (Lemos et al., 2015)

The timescale of the process of light absorption, fluorescence and phosphorescence is on the order of femtoseconds (10^{-15} s), pico- to nanoseconds, and microseconds to seconds, respectively. The measurement of

how long a molecule spends in the excited state is of interest as it helps provide further information. Both the spectral position of fluorescence and the excited state lifetime are dependent on the fluorophore, its interaction and the local environment. The fluorescence lifetime can be thought of as the molecule's average period of time spent in the excited state, or better defined as how long an excited ensemble of molecules takes to decay to 1/e of its initial intensity. The excited state decays in an exponential manner and it can be described by the equation (Eq 5.1):

$$I(t) = I_0 e^{-t/\tau}$$

Equation 5.1

where τ is the fluorescence lifetime. Fluorescence lifetime is an absolute measurement, whereas the steady state intensity is a relative measurement in contrast with the steady state, which is a relative measurement. Additionally, fluorescence lifetime is independent of concentration (within certain limits), since is an intrinsic molecular property.

As fluorescent molecules can be highly affected by the conditions of their environment and/or the presence of other interacting molecules, it is possible to use fluorescence lifetime to help elucidate information regarding changes of the local environment, e.g. pH, viscosity, polarity etc., molecular interactions, size and shape of molecules, inter- and intramolecular distances, kinetic and dynamic rates, and resolution of molecular mixtures.

Because of the specificity of the fluorescence lifetime, scattered excitation and background fluorescence can be easily distinguished. Moreover, Forster Resonance Energy Transfer (FRET), quenching, and fluorescence anisotropy measurements are easily determined using fluorescence lifetime; enabling more parameters to be recovered. However, it should be noted that steady state and time-resolved measurements are complementary in clarifying as much of the fluorescence signature as possible.

Time-correlated single-photon counting (TCSPC) is the most sensitive method for determining picoseconds to microseconds fluorescence lifetimes. The basis of TCSPC is that the probability to detect a single photon at a certain time after an excitation pulse is proportional to the fluorescence intensity at that time. To

make the process more intuitive, as the decay appears linear, a log intensity axis is commonly used, making data interpretation easier as a single exponential decay will appear linear. Interactions can result in the “quenching” of the fluorescence, which can be either dynamic (change in lifetime and intensity) or static (change in intensity only). This can happen by molecular binding, electron or energy transfer (e.g., FRET) or another environmental change. The exact emission behaviour depends on the nature of the fluorophore. The decay can become multiexponential, i.e., the states have different lifetimes, and this can happen if there are different populations of the same molecule showing different interactions/environments causing differences in the excited state.

Time-resolved emission spectra (TRES) can be used for the resolution of emitting species, with different lifetimes and overlapping spectral properties (Fig 5.2). A 3-D surface of intensity-time-wavelength is obtained, and analysis can be performed by simply taking slices at different times to see the evolution of the spectral shape. A global analysis can also be performed in order to obtain the lifetimes from the fluorescing species. Further analysis of these lifetimes can be used to recover decay associated spectra, i.e. spectra relating to the different lifetimes.

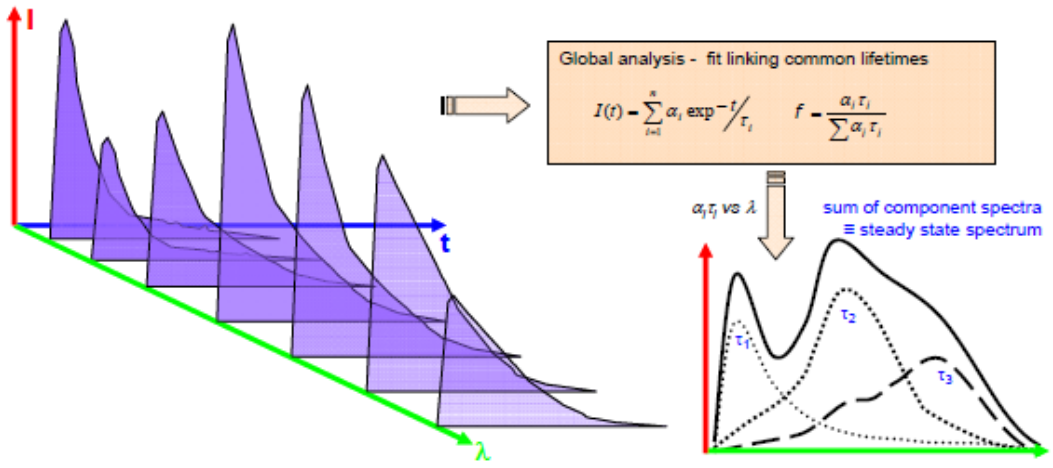


Figure 5.2 Time-resolved emission spectra (TRES) illustrating time slicing at two points and production of decay associated spectra

The measurement of fluorescence anisotropy is another commonly used method. This polarisation measurement is usually employed in order to reveal

viscosity information, although by the use of molecular rotors a simpler lifetime measurement can also be obtained (Kuimova *et al.*, 2008; Hungerford *et al.*, 2009). Steady state anisotropy provides one measure (r) only, whereas time-resolved allows more information to be acquired, as shown in the following formula (Eq 5.2):

$$r(t) = r_\infty + (r_0 - r_\infty)\exp(-\frac{t}{\tau_r})$$

Equation 5.2

where *r* is the anisotropy, r_∞ is the anisotropy at infinite time, r_0 is the initial anisotropy, τ_r is the rotational correlation time, which can be determined and related to the effective volume of a molecule (*V*) and the local viscosity (η) (Eq 5.3). Also, *T* is absolute temperature and *k* is Boltzman's constant. Note if the viscosity is known then the rotational correlation time can be used to monitor changes in the effective volume of a molecule. This can have application if a protein changes shape or fragments / aggregates.

$$\tau_r = \frac{V\eta}{kT}$$

Equation 5.3

Here, an objective of the use of fluorescence, and in particular time-resolved fluorescence, is to ascertain if it can elucidate whether ultrasound treatment causes any change in enzymatic structure. Although extracted enzymes used in this study are in fact mixtures of compounds originating from the plant employed, in this study purified enzyme was used. This "simplifies" the interpretation of the fluorescence and narrows the investigation to the active molecule of interest, i.e. the enzyme. Thus, purified horseradish peroxidase (HRP) and polyphenoloxidase (PPO) were purchased and used as received.

Native fluorescence has previously been employed to examine HRP (Pappa and Cass, 1993; Das and Mazumdar, 1995a; Tsaprailis, Sze Chan and English, 1998). Principally alterations in enzyme structure or conformation influence the fluorescence emission of the Tyr and Trp residues because of distance facilitated interactions, e.g. Forster mechanism energy transfer. Additionally, a similar interaction between them and the haem group, allows monitoring any structural modifications, using the fluorescence emission from these amino

acids. Application of light at 272 nm enables both Tyr and Trp moieties to be addressed (Lakowicz, 2006) and the overall fluorescence demonstrates a hypsochromic shift as opposed to using a wavelength (i.e. 295 nm) specifically to excite the Trp (Das and Mazumdar, 1995a) and is associated to the Tyr contribution. Even though Trp emission is usually employed, Tyr emission is also informative towards changes in protein structure (Zhdanova *et al.*, 2015). The presence of haem usually results in quenching of tryptophan's fluorescence (Ohlsson *et al.*, 1986), while the latter amino acid can cause Tyr emission to be quenched (Lasagna *et al.*, 1999). Fluorescence quenching is distance related, thus conformational modifications in both protein structure and the active site of HRP can be determined. The fluorescence of PPO is more complicated because of the larger quantity of fluorescent amino acids within its structure, which gives more of a possibility for energy transfer interaction between the tyrosine and the tryptophan. However, as with HRP it is possible to make use of these amino acids to elucidate change within the protein structure, although the fact that there are many fluorescent residues means that it can be advantageous to make use of an external fluorescent label. This can be chosen to absorb and emit light at longer wavelengths than the native fluorophores, thus simplifying the interpretation of the fluorescent signal.

The relative position of these fluorescent amino acids in relation to the active site can be seen, in Fig 5.3. In the smaller HRP basic molecular modelling showed a range of Tyr-Trp distances in the order of 1.3 nm to 3.5 nm, while Tyr haem distances were in the range ~0.9 nm to 2.5 nm.

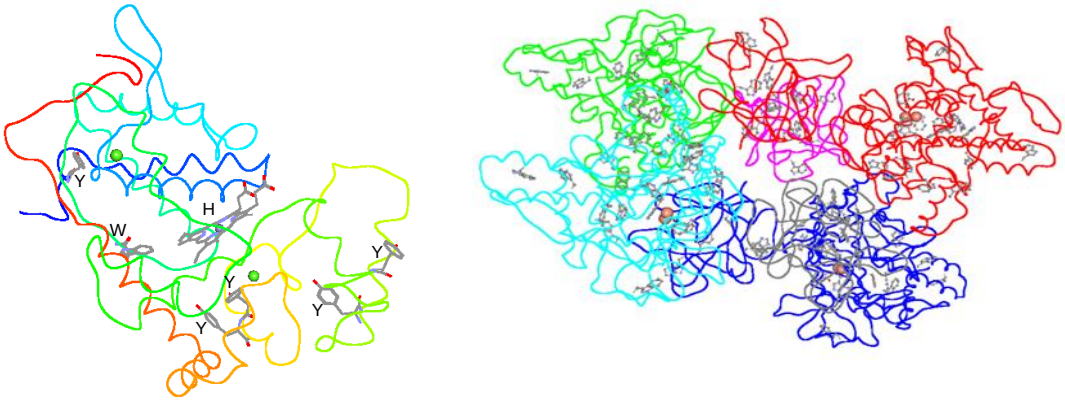


Figure 5.3 Cartoon representation (not relative scale) of (left) HRP structure showing the tyrosine (Y-7,185,201,233,234), tryptophan (W-117), haem (H) and Ca atoms (green spheres) (Tsikrika et al., 2017); (right) PPO structure showing its tetrameric form. Tyrosine and tryptophan residuals, along with copper atoms are also displayed.

The distance from the Trp to the haem was estimated as 1.3 nm, and it is in accordance with the literature (Pappa and Cass, 1993). Hence, energy transfer between Tyr and Trp is within the reported critical transfer distance (distance at which energy transfer is 50% efficient) i.e. up to 2 nm (Kronman and Holmes, 1971). Unlike HRP, which has a single tryptophan, PPO is more complex, with a larger number. This makes interpretation of the fluorescent signal more difficult, also there are reports that its tetrameric structure can fragment (Ismaya *et al.*, 2011) which may influence its fluorescent behaviour.

In this section time-resolved techniques, principally the use of decay associated spectra were used to monitor the influence of US on the native fluorescence behaviour of the two enzymes. In the case of PPO an external fluorescent label was employed to see if US caused any fragmentation of the tetrameric structure by the measurement of time-resolved fluorescence anisotropy.

5.2 Materials and Methods

5.2.1 **Chemicals**

Peroxidase from horseradish (HRP, EC 1.11.1.7) and tyrosinase from mushroom (EC 1.14.18.1), bovine serum albumin (BSA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Gillingham, UK). FUN-1 label was purchased from Fisher Scientific (Loughborough, UK).

5.2.2 **Enzyme solution**

Aqueous solutions of 0.005 mg/mL of horseradish peroxidase (HRP) and 0.004 mg/mL of polyphenoloxidase (PPO, tyrosinase from mushroom) were prepared with distilled water.

5.2.3 **Ultrasound treatments**

5.2.3.1 Sonication of HRP

Ultrasound was applied for 60 min using a Meinhardt Ultraschlltechnik high frequency sonicator operating at 378 kHz and 32W, 583 kHz and 34 W, or 583 kHz and 48 W. During the experiment, a cooling jacket connected to a

cryostatic bath (Fisher Scientific ISOTEMP Thermostatic) controlled the temperature. Samples were withdrawn after 2, 4, 6, 8, 10, 15, 30, 45, and 60 min of sonication and kept in ice until they were analysed.

5.2.3.2 Sonication of PPO

Ultrasound was applied at 583 kHz and 48 W for 75 min using the Meinhardt Ultraschlltechnik high frequency sonicator, while a cooling jacket connected to a cryostatic bath (Fisher Scientific ISOTEMP Thermostatic) controlled the temperature. Samples were withdrawn after 5, 10,15, 20, 25, 30, 40, 50, 60, and 75 min of sonication and kept in ice until they were analysed.

5.2.4 **Thermal treatment**

A 250 mL Duran glass bottle containing 200 mL of PPO solution was placed in the middle of a water bath previously equilibrated at 40^{o}C. At predetermined time intervals (5,10, 15, 20, 25, 30, 40, 50, 60, and 75 min) samples would be removed of the bath and then they would immediately be immersed in an ice bath and kept there prior to analysis.

5.2.5 **Fluorescence**

Time-resolved measurements were performed using a Horiba Scientific DeltaFlex (Fig 5.4) with DeltaDiode excitation (DD-270) at 272 nm. At this excitation wavelength both tyrosine and tryptophan residues would be excited. Decay associated spectra were obtained over the wavelength range 295 nm to 425 nm, with decay data collected at 5 nm intervals. These data were obtained by measuring the time-resolved fluorescence decay for a fixed time at a particular wavelength, then increasing the wavelength and repeating the measurement process. The resulting dataset was analysed globally with decays modelled by five common decay times in all cases. This was required in order to obtain a good fit and enabled relative changes to be seen between the untreated and treated samples. Excitation-emission matrices (EEMs) scanning the emission spectra for different excitation wavelengths were recorded using a FluoroLog 3. Auxiliary information was obtained by molecular modelling made

using ArgusLab (Plenaria Software) by rendering a structure from the RCSB Protein Data Bank (pdb1gwu).

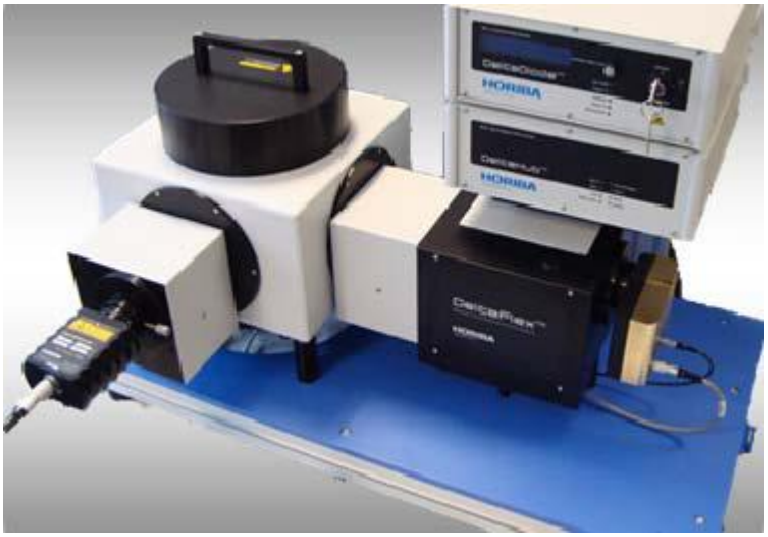


Figure 5.4 Horiba Scientific DeltaFlex

5.3 Results and Discussion

5.3.1 **Effect of ultrasound treatment on HRP**

Fluorescence excitation-emission matrix (EEM) scans of the HRP both untreated and ultrasound treated at 378 kHz and 32 W are shown in Fig 5.5. Rayleigh and Raman scattering features are evident in the EEMs, caused by the solvent, due to the low concentration of the enzyme. It can also be seen that ultrasound treatment caused a remarkable change in the wavelength of the principal emission from ~335 nm to ~407 nm. This wavelength is not normally attributed to the presence of the principal fluorescent amino acids (Tyr and Trp) of HRP. Additionally, there is an alteration in the excitation wavelength, which in combination with the emission data, suggest the formation of a new species.

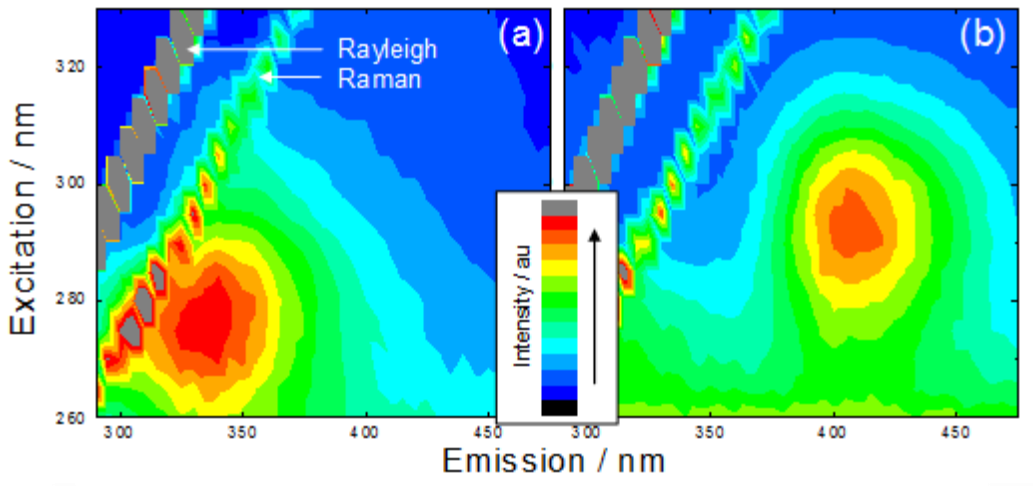


Figure 5.5 EEMs of (a) untreated and (b) ultrasound (378 kHz) treated HRP solution

Similar data was obtained using 583 kHz ultrasound treatment (Fig 5.6). Rayleigh and Raman scattering features can be seen on the left of the EEM, again caused by the solvent. Furthermore, the wavelength of the principal emission moved to ~407 nm, while it is also shown that there is a shift in the excitation wavelength, supporting the evidence of the presence of a new species.

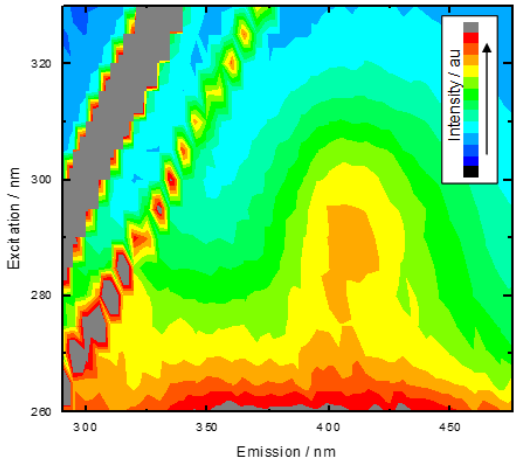


Figure 5.6 EEM of ultrasound (583 kHz) treated HRP solution

These observations were further explored using time-resolved spectroscopy. The same excitation wavelength of 272 nm was chosen again, as this should excite the newly formed species (although not as strongly as if the optimum wavelength of 292 nm was employed), as well as the native fluorophores present (i.e. Tyr and Trp). According to literature, at 280 nm (close to excitation wavelength chosen in the present work) the extinction coefficients of Tyr and Trp are 1480 M^{-1} cm^{-1} and 5540 M^{-1} cm^{-1} respectively (Mach, Middaugh and

Lewis, 1992). Additionally, Tyr outnumbers Trp seven to one, so it is expected nearly twice as much light to be absorbed by Tyr as opposed to Trp. The choice of excitation wavelength should also enable any changes in HRP structure to be deduced. A Time-Resolved Emission Spectrum (TRES) measurement was performed on the samples, analysis of which provide decay associated spectra to be obtained (Fig 5.7). This analysis allows the lifetime to be associated with spectral shapes and is a useful technique to separate mixtures of fluorophores.

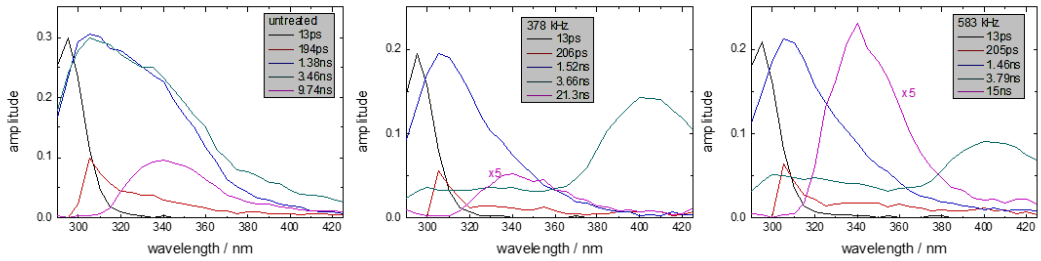


Figure 5.7 Decay associated spectra for untreated and ultrasound treated (60 min.) HRP in deionised water. The excitation wavelength was 272 nm.

Time-resolved fluorescence measurements of HRP revealed the presence of a spectrum relating to a short-lived component in all three of the samples and can easily be attributed to Raman scattering from the solvent. Moreover, the presence of an ill-defined spectrum associated with a lifetime of ~200 ps is clear, with a minor contribution to the overall emission. A similar lifetime component has been previously reported in the apoprotein of HRP, which could be suggestive of a partial disappearance of the haem group (Das and Mazumdar, 1995a). The removal of an energy transfer pathway from the Trp (Ohlsson *et al.*, 1986) can result in the increase (Das and Mazumdar, 1995a) of the shorter-lived fluorescence lifetime component (of the 3 exponentials present) (Das and Mazumdar, 1995b). However, those studies concerned excitation of the Trp, specifically, whereas, in the present study, Tyr is also excited. Two sub nanosecond decays were recorded in another study using 280 nm excitation, but longer decays on the nanosecond timescale were out of their time window (Neves-Petersen *et al.*, 2007). Trp and Tyr when both free in solution, exhibit dominant decay associated spectra with a lifetime of 3.4 ns, with minor contributions of 0.54 ns and 0.98 ns (Trp and Tyr respectively) (Lakowicz, 2006). Therefore, when considering the untreated sample, it is expected that the other decay associated spectra resemble a combination of

both their emissions, with a longer-lived (9.47 ns) spectrum solely ascribed to Trp. However, it should also be noted that at the excitation wavelength used here, in addition to direct excitation of each amino acid, an augmentation in Trp emission is made via energy transfer (Lasagna *et al.*, 1999). Hence the combined spectra (1.38 ns and 3.46 ns components) may also be indicative of this interaction between these two species.

Time-resolved data reveal that ultrasound treatment results in a significant change in the spectral behaviour. Although this is also evident in the EEM's, separation of the individual fluorescing components is not so apparent. On the other hand, a longer-lived associated spectrum only attributed to Trp is still present. However, this is a minor contribution and its lifetime is increased in relation to the untreated sample. It should be pointed out that Trp emission in proteins can be multi-exponential in nature and component lifetimes as long as 16 ns have been reported (Schauerte and Gafni, 1989).

The shorter wavelength emission (associated with an ~1.4 ns decay) is more indicative of Tyr in shape, while the emission around 407 nm is more noticeable (in accordance with the EEM), with an associated decay time of ~3.7 ns. Trp and Tyr relative emissions differ as well, while there is a dramatic decrease in the enzymatic activity (residual activity in both cases ~3%). Analysis of the absorption spectra (Fig. 5.8) indicates the removal of the haem (Tsaprailis, Sze Chan and English, 1998), which could also explain the decrease in activity.

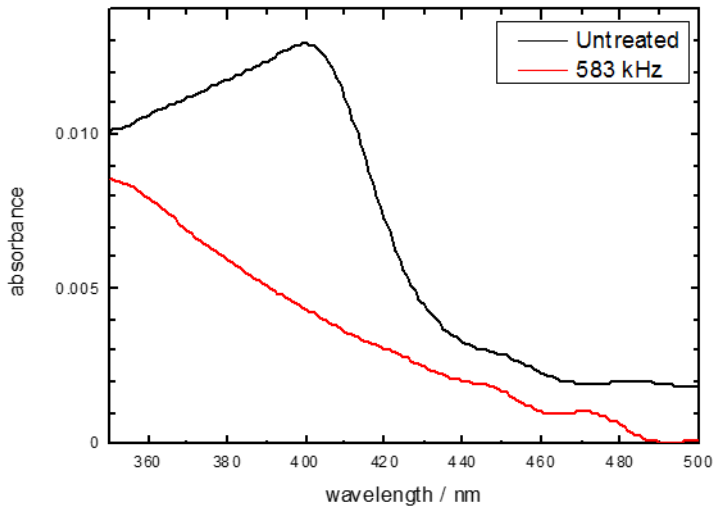


Figure 5.8 Absorption spectra (in region of haem absorption) for untreated and treated (in this case 583 kHz for 60 min.) HRP in solution.

Previous work on HRP has observed an increase in Trp emission when it is excited directly (Ohlsson *et al.*, 1986). Moreover, denaturing studies on HRP followed by fluorescence have ascribed this to initial alterations in secondary structure followed by removal of the haem (Pappa and Cass, 1993). In the present work, even though there is direct excitation of both Trp and Tyr, a similar behaviour is expected. The decay associated spectra after ultrasound treatment show clear individual Tyr and Trp emission shapes. A decrease in the relative contribution from the Trp emission is also observed, in contrast to the combined shape present before treatment. This finding is in accordance with the fact that conformational change in the HRP structure is increasing Tyr-Trp distances and decreasing the contribution of Trp emission obtained via energy transfer. Tyr residues are outnumbered to those of Trp ones, consequently it is expected that the latter would absorb less of the excitation light. This combined with a decrease in energy transfer to the Trp, leads to a relative increase in their contribution to the overall fluorescence in comparison to the Trp emission. This route would seem to have more of an impact than any increase in Trp fluorescence associated with haem removal and may suggest a significant alteration in the HRP secondary structure. A similar increase in shorter wavelength (Tyr) emission has previously been reported by Tsaprailis, Sze Chan and English (1998) using excitation at 280 nm examining denaturing of HRP by guanidine hydrochloride (GdHCl). They postulated a two-step unfolding

process, while changes in the secondary structure and loss of haem, followed by a second transition associated with the Trp chain were also observed. Although the removal of the haem and alterations in protein structure affect the Tyr and Trp fluorescence behaviour, it does not explain the emission around 407 nm. HRP has previously been used to act as a catalyst on tyrosine containing substrates in the presence of hydrogen peroxide for the formation of di-tyrosine (Tang *et al.*, 2006). This species (Fig 5.9), is reported to have a fluorescence emission at 407/408 nm (Huggins *et al.*, 1993; Tang *et al.*, 2006) and formed via a metal-catalysed oxidation in the presence of H_2O_2 (Tang *et al.*, 2006).

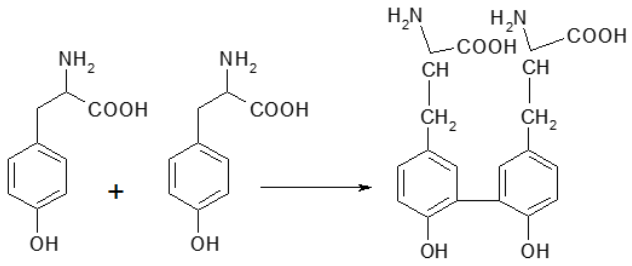


Figure 5.9 Representation of the formation of di-tyrosine from tyrosine catalysed in the presence of a metal and hydrogen peroxide.

Both components (metal and H_2O_2) can be readily found in an aqueous solution of HRP treated with US. The metal is present in the haem moiety of HRP while H_2O_2 can be produced by the dimerisation of $OH^{\bullet}$ during bubble implosion caused by the application of ultrasound to the water (Islam *et al.*, 2014). Thus, it can be possible that such a mechanism may be in action here. The proximity of Y-233 and Y-234 (Fig 5.3) could be indicative of the origin of the longer wavelength emission. However, an interaction involving Y-185 or Y-201 could also be the case. An estimation by molecular modelling shows that these residues are separated by ~1.3 nm, while the distance between Y-201 and Y-233 is estimated to be ~1.0 nm. In the case of the non-neighbouring residues to be the reactants this would also imply that a very significant change in the HRP secondary structure had occurred.

In summary, the application of ultrasound treatment has had a detrimental effect on HRP. Because the temperature was controlled during sonication treatments this impact cannot be ascribed to thermal denaturing of the enzyme. Additionally, time-resolved fluorescence has allowed the identification of

changes via the formation of a new fluorescing species constituting a means to acquire further insights on how ultrasound can influence enzyme behaviour. However further investigation is needed in order define the effect of different acoustic powers and treatment times.

5.3.2 **Effect of ultrasound treatment on PPO**

In a similar way to the HRP study, PPO was investigated. However, in this case both steady state and time-resolved fluorescence measurements were made on aliquots taken at fixed time intervals during the ultrasound treatment. Similarly, a control sample of enzyme undergoing a thermal treatment at 40 ºC to mimic the heating effect of the ultrasound treatment was also measured. The steady state spectra obtained from for both the ultrasound and thermally treated samples over a 75-minute period are shown in Fig 5.10.

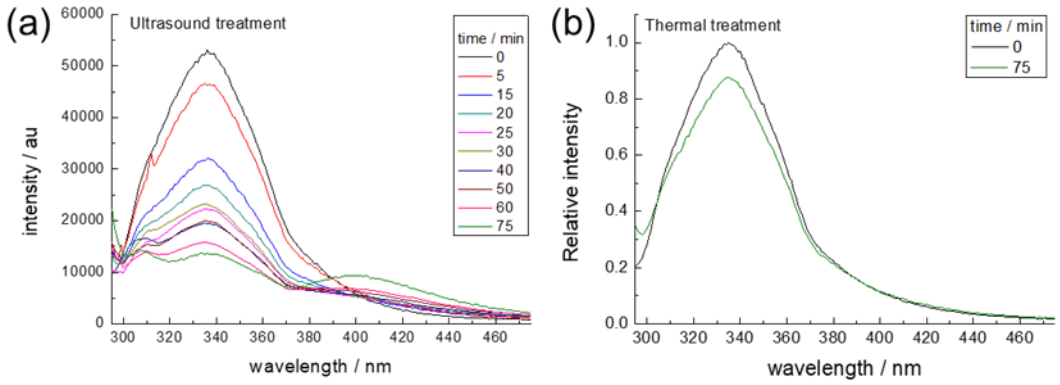


Figure 5.10 Steady state fluorescence spectra excited at 280 nm for (a) ultrasound and (b) thermally treated PPO.

It can clearly be seen that there is a marked decrease in the main emission band for the ultrasound treated sample. Also, there is a growth in an emission just over 400 nm. However, there is only a small decrease in the emission upon thermal treatment (equivalent to 5 minutes of ultrasound) and no sign of the longer wavelength growth, which can therefore be ascribed to the effect of the ultrasound treatment.

Part of the objective of this study is also to see if fluorescence can be used as an indicator of the enzyme activity. To this end, although a comparison can be made with the activity measurements of the extracted enzyme for completeness

the activity of pure PPO was measured, and the residual activity was calculated the same way as described in section 4. The outcome of the thermal and ultrasound treatments on the residual activity is shown in Fig 5.11.

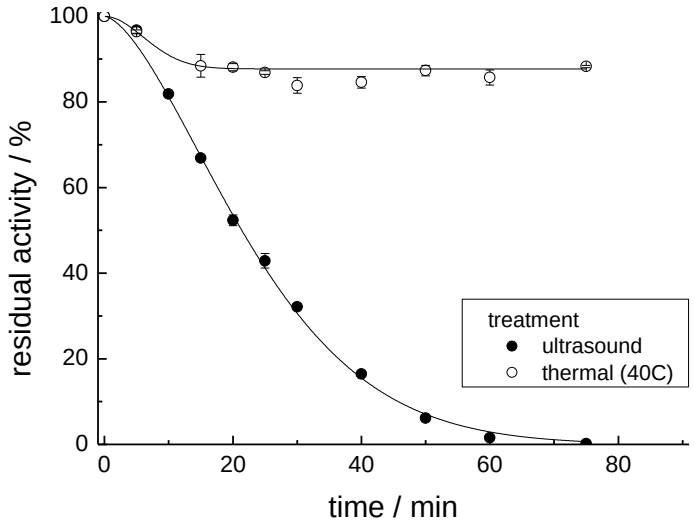


Figure 5.11 Residual activity for ultrasound and thermally treated PPO. The data has been fitted using a Weibull model.

The behaviour of the thermally treated pure sample is similar to that seen in section 4, with the residual activity after 75 minutes (~87%). It should be noted that this corresponds to the change in the relative peak intensities seen in the fluorescence emission (Fig 5.10). Thus, showing the potential for fluorescence to be an indicator for the activity. The residual activity data for the pure PPO (0.2% after 75 min of US treatment) is also in keeping with that of the extract (3.6% after 75 min of sonication). In order to analyse the correspondence with the fluorescence data a plot of the change in the relative peak intensity (at 335 nm) was made (Fig 5.12). This was also fitted to a Weibull model (solid line fit), but the values obtained (d=-0.92, k=0.091) do not correspond to those of the residual activity data. The plot is clearly not linear, however could be considered as two linear regions (dotted line fit), with a transition at ~25 ºC.

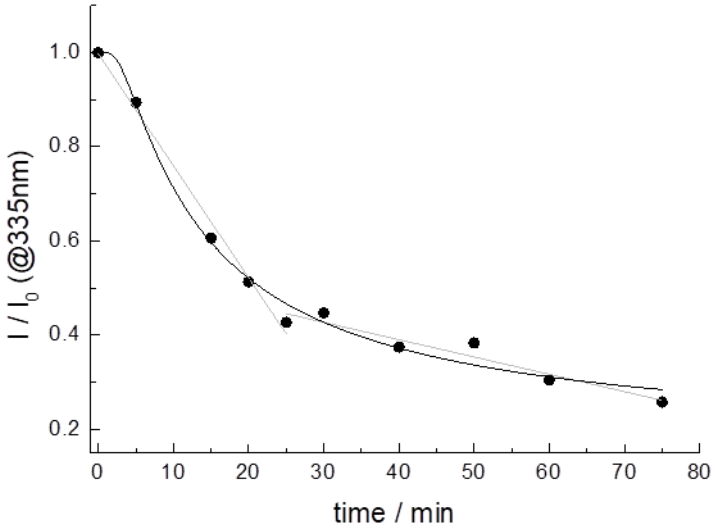


Figure 5.12 Relative change in fluorescence for ultrasound treated PPO. The data has been fitted using a Weibull mode (curve) and two linear regions.

Just considering this dataset, it is not possible to determine which is the "correct" model for this data and it should be kept in mind that, with this dataset, not only was there a decrease in the peak but an increase at a longer wavelength (~400 nm). The fact that another fluorescing species is present can easily mean that there would be a deviation from the relationship between fluorescence and residual activity potentially seen for the thermally treated sample. The growth in the longer wavelength emission can be seen more clearly when normalising to the 335 nm peak emission (Fig 5.13).

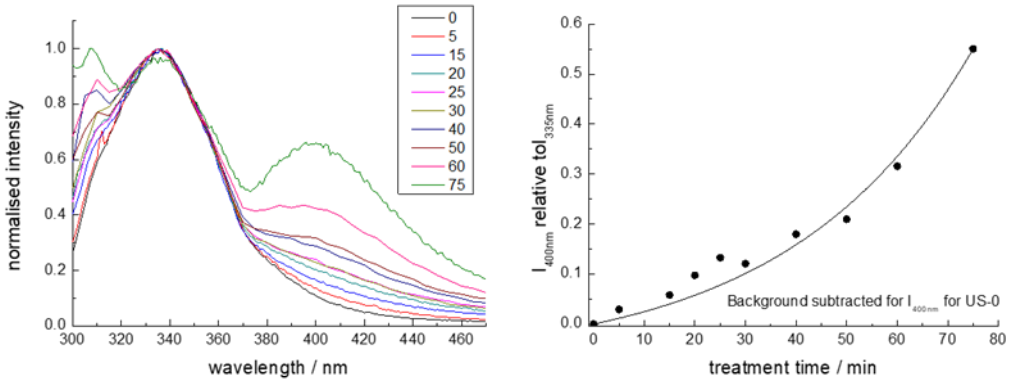


Figure 5.13 Relative change in the longer (~400 nm) fluorescence emission for ultrasound treated PPO. The data has been normalised to the peak emission at 335 nm.

Fig 5.13 shows that at the end of the ultrasound treatment (75 min) the newly formed longer wavelength emission makes a very significant contribution to the overall fluorescence emission. Although it is tempting to attribute this emission

in a similar way to that in HRP further investigation is required. However, what is clear is that fluorescence is a very good technique to show the influence of the ultrasound process with a decrease in tyrosine / tryptophan emission and the increase in a new emissive species.

As with the HRP samples, decay associated spectra were obtained (Fig 5.14).

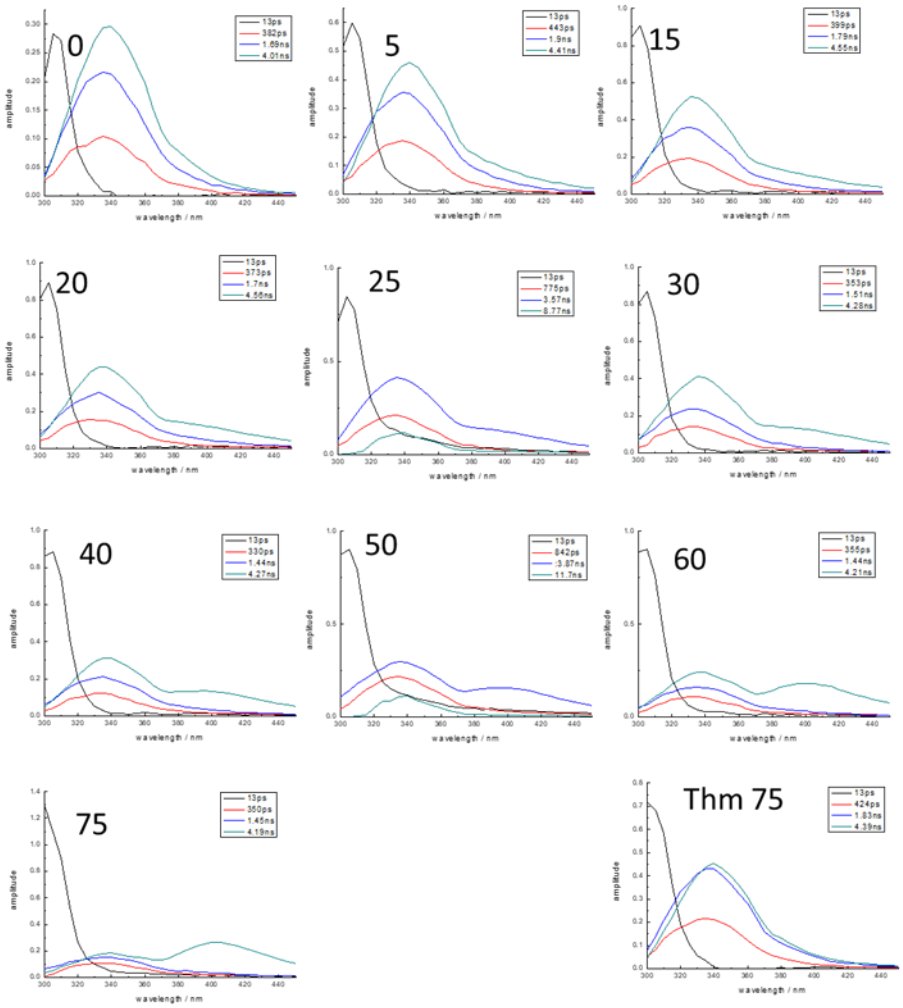


Figure 5.14 Decay associated spectra for ultrasound treated PPO at different treatment times. The decay associated spectra for a sample thermally treated for 75 minutes (Thm 75) is also given.

The decay associated spectra show the presence of a short-lived component (fixed at 13 ps) which can be ascribed to Raman scattering from the solvent. Since there are a much larger number of fluorescent tryptophan and tyrosine residues in PPO as compared to HRP the interpretation is more complex and the spectral and time-resolved properties will reflect an “average” of the different fluorescing species (or environments). The emission from tryptophan (both direct and through energy transfer) can be ascribed to the emission close to

335nm), with tyrosine emission appearing as a shorter wavelength shoulder. However, it is clear from the decay associated spectra that the longer wavelength (~400 nm) emission is associated with a lifetime of ~ 4 ns and that its contribution starts to become significant after a treatment time of 25 minutes. This would tie in with the use of two linear regions when looking at the change in fluorescence intensity (Fig 5.12). It is also noteworthy that the thermally treated sample does not show any longer wavelength emission, thus again showing that this is an effect of the ultrasound treatment. Whether the origin of this emission is the same as that seen in HRP is open to discussion, as there are slight differences in wavelength and lifetime and the more complex structure of the PPO. However, it is clear that fluorescence can sense a change induced by the US treatment.

It is known that PPO can exist as a tetramer (Kanteev, Goldfeder and Fishman, 2015) and there is the possibility that fluorescence anisotropy can be used to see if changes in molecular volume (e.g. by fragmentation of the protein) occurred during the ultrasound treatment. This can be done since the rotation correlation time (obtained by analysis of the time-resolved fluorescence anisotropy) is proportional to the effective molecular volume (Eq 5.3). However, to obtain good anisotropy data the fluorescence lifetime should be similar in magnitude to the rotational correlation time, as not less than a tenth of it (Lakowicz, 2006), because of the complexity of the native fluorescence and the fact that it is relatively short-lived in comparison to the expected rotational correlation time. A molecular volume of 357 nm^3 was estimated from molecular modelling, which implies a rotational correlation time in the order of 79 ns. This means that an external fluorescent label is required to attach to the PPO to make this measurement. Another complication is that covalent linking protocols for label attachment use buffer conditions (pH 9, for example) that can themselves cause changes in the enzyme structure. Therefore, a label that would attach in an electrostatic manner or partition into the enzyme because of hydrophobicity was chosen. Out of the labels available for use FUN-1 was selected. The structure is shown in Fig 5.15.

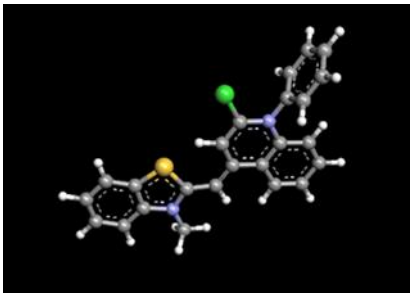


Figure 5.15 Representation of the structure of the FUN-1 fluorescent label.

This label has been used as with yeast (Essary and Marshall, 2009; Holmes-Smith *et al.*, 2013). Although not ideal, as its the fact that its lifetime is around 4 ns (Holmes-Smith *et al.*, 2013), which is a little short for this application, it was found to be applicable for use with PPO and its excitation wavelength of 496 nm means that no native fluorescence from the enzyme should be excited.

To try and overcome the limitation of the relative short lifetime of FUN-1 with a molecule with a longer rotation correlation time, anisotropy measurements were taken to a lot greater precision (number of counts in the peak of the decay) than normal. 200,000 counts were used, as opposed to the usual 10,000. An initial measure to check the ability of this label was made using a known sample (BSA). This has a molecular volume of 163 nm^3 (Flecha and Levi, 2003). A small volume of FUN-1 in DMSO was added to 1 mg/mL of BSA in water. The results obtained gave a rotational correlation time of 35.6 ns, which returns a volume of 162 nm^3, which is in good keeping with the literature value. Therefore, this approach may hold promise for use with the larger PPO molecule, although not optimum in terms of the label lifetime, which should preferably be longer.

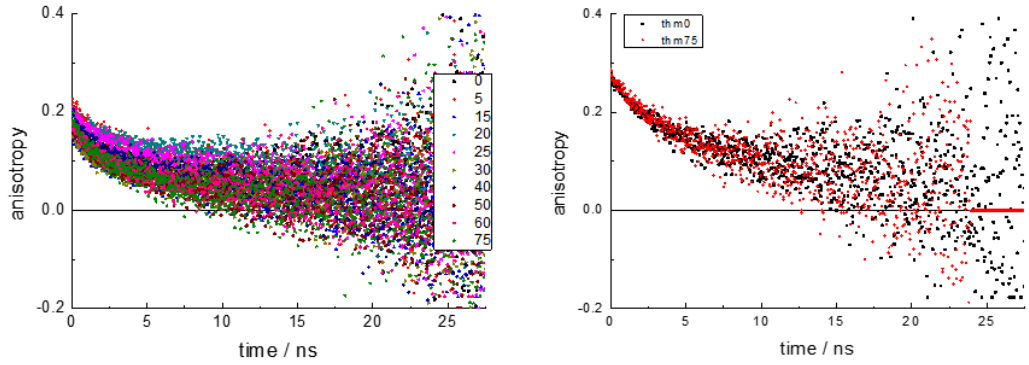


Figure 5.16 Representation of the anisotropy decay curve sat different times (minutes) for ultrasound (left) and thermally (right) treated PPO.

FUN-1 was added in DMSO to samples taken at different times after treatment. The anisotropies for the different US treated samples were measured along with that of the thermally treated (for 75 minutes) sample and the anisotropy curves are shown in Fig 5.16. The resultant anisotropy decay curves for the ultrasound treated sample are indicative of a change in the anisotropy having occurred with the US treatment. On the other hand, the sample which had the thermal treatment for 75 minutes appears to be similar to that obtained for untreated PPO. Again, these results are indicative that there is an influence of US treatment on the PPO structure, which is not related to thermal heating of the sample during the application of ultrasound.

The data were further analysed to determine the rotational correlation time and then this was related to the molecular volume. Fig 5.17 shows that two rotational correlation times were required to get a satisfactory fit to the data. The shorter one may relate to some local mobility of the label in the enzyme but should not relate to “free” label in solution as this would be expected to be much faster (<100 ps). The longer rotational can reflect the motion of the whole enzyme.

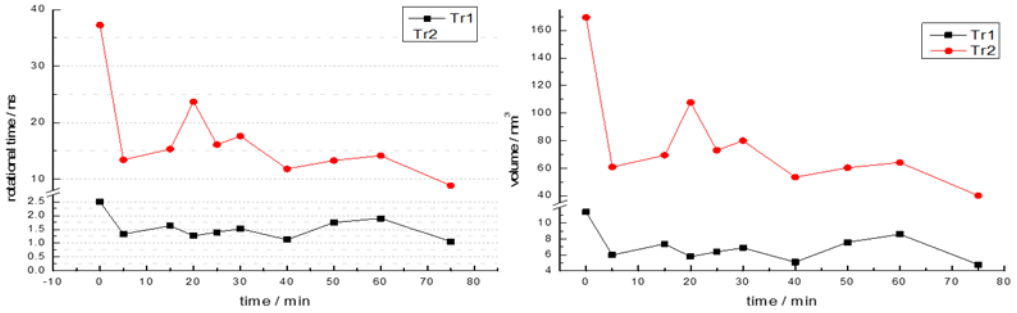


Figure 5.17 The determined rotational correlation times (left) and the effective volumes calculated from them (right) for ultrasound treated PPO.

Both the rotational correlation time and hence the effective volume display values that are smaller than those expected initially for PPO. From molecular modelling a volume in the order of 357 nm^3, which relates to a rotational correlation time of 79 ns were obtained. Thus, these results need to be treated with caution. Things which need to be taken into account are the fact that these measurements were performed after the other fluorescence measurements, which may give time for sample degradation. Also, the label lifetime is not optimum, although the initial test with BSA proved promising the lower concentration of PPO meant that a smaller quantity of label was used leading to longer experimental run times in order to get the precision in the measurements – another possibility for sample degradation. The label may also not bind so well to PPO as compared to BSA. Thus, further investigation is required. However, in spite of this and although the values are not as expected, there does appear to be some change in the sample as indicated by difference in anisotropy. To see if changes could be further elucidated by looking at relative changes a plot of the relative change in effective volume was made for the ultrasound treated samples. As the PPO is a tetramer if it were to fragment it would most likely do this by either forming a dimer or a monomer. Hence relative changes in molecular volume should be quantised. The plot of this is shown in Fig 5.18 and shows that this may, in fact, be the case.

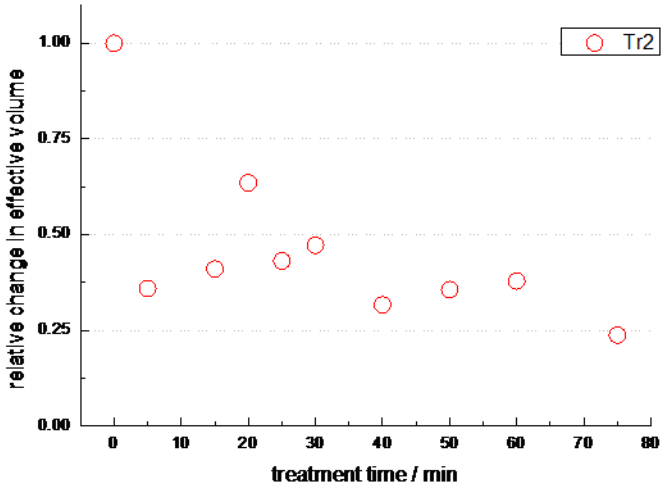


Figure 5.18 The relative change in effective molecular volume, obtained by considering the longer rotational correlation time for ultrasound treated PPO.

It is acknowledged that the anisotropy results should be treated with some caution as further investigation is required. However, even with the results so far obtained it is clear that this is a very promising method by which to study the effect of ultrasound on the PPO structure.

5.4 Conclusion

It is clear that making use of fluorescence is a power means to follow the effect of ultrasound treatment on the two enzymes used in this study. It shows that changes in enzyme structure are occurring which are not produced by the thermal heating when ultrasound is applied. There is the apparent formation of a new fluorescing species, possibly mediated by the formation of hydrogen peroxide caused by radical formation as the bubbles implode. The use of decay associated spectra can distinguish this. Although promising the use of fluorescence anisotropy in the case of PPO requires further optimisation and investigation as it could provide a good means to assess changes in the tetramer structure.

6 Effect of Ultrasound on Enzyme Activity and Bioactive Compounds of Strawberry Puree during Storage

6.1 Introduction

Strawberries (*Fragaria x ananassa*) are one of the most popular fruits worldwide because of their unique flavour and taste. They can be consumed fresh, processed into juices or jams, or added to yogurts or other desserts (Terefe *et al.*, 2010a). Strawberries are rich in phenolic compounds including flavonoids (Fig 6.1) such as anthocyanins (mainly pelargonidine-3-glucoside and cyanidin-3-glucoside), flavonols (quercetin, kaempferol, myricetin), flavanols (catechins and epicatechin), hydrolysable tannins (ellagitannins), and phenolic acids (Seeram, 2006).

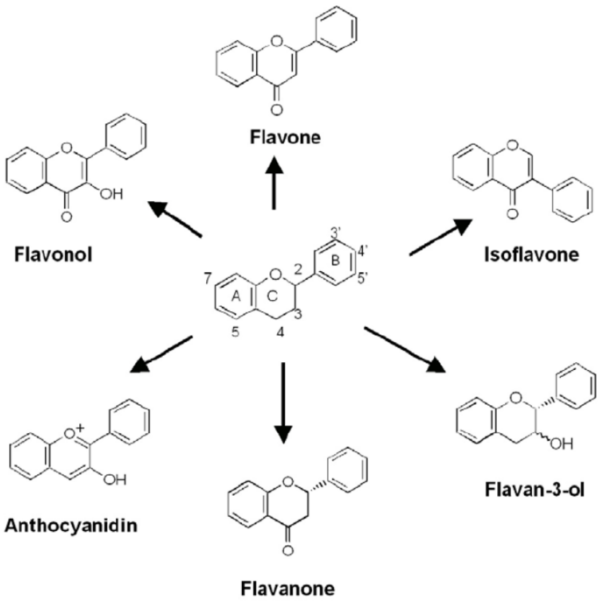


Figure 6.1 Basic structures of flavonoids (Nishiumi et al.*, 2011)*

Studies performed *in vitro* and *ex vivo* using strawberries, amongst other fruits, suggest that the aforementioned phytochemicals are involved in a variety of biochemical reactions exerting antioxidant and subsequently immunomodulating, inhibition of platelet aggregation and anticarcinogenic action (Azzini *et al.*, 2010). However, strawberries are quite sensitive to colour, texture, and flavour deterioration, mainly due to fungal attack and oxidative enzyme activity, such as polyphenoloxidase (PPO), and peroxidase (POD).

PPO and POD are involved in browning reactions, associated with discolouration and decrease in flavour (Lopez *et al.*, 1994) and consequently with quality loss of the nutritional and market value of food and food products (Cheng, Zhang and Adhikari, 2013). Additionally, studies have shown that these two enzymes are also responsible for the degradation of anthocyanins and other polyphenols in many fruits, including strawberries (López-Serrano and Ros Barceló, 2002; Chisari, Barbagallo and Spagna, 2007). Therefore, the inactivation of these enzymes is necessary, in order to extend the shelf life and ensure the quality of strawberries, as well as their juice.

Thermal processing technologies, which are mainly used for enzyme and microorganism inactivation, have detrimental effects on the physicochemical and nutritional quality attributes of strawberries, such as loss of nutritional components and alterations of colour, flavour and texture (Zabetakis *et al.*, 2000; Patras *et al.*, 2009). An alternative technique for processing and preservation of fruit and vegetable products is ultrasound (US) and it has been used in the food industry for cooking, cutting, emulsification/ homogenisation, crystallisation, extraction and microbial inactivation (Pingret, Fabiano-Tixier and Chemat, 2013). Applications of US in food processing have been reviewed by Knorr *et al.*, (2004), while effects of US on fruit juices have been recently reviewed by Zinoviadou *et al.* (2015), and Tiwari *et al.* (2008). These reviews point out that the effect of US is very much depending on the food matrix, thus it is essential to assess each product before it can be marketed.

As mentioned before, high power US propagating in a liquid, induces bubble cavitation, causing high shearing effects and localised high temperatures around 5000 K and pressures up to 50,000 kPa. The intense local energy and high pressure facilitate localized pasteurisation, without causing a significant rise in macro-temperature (Tiwari, O'Donnell and Cullen, 2009b). Other effects caused by the US include mechanical stresses produced by microstreaming, implosion shock waves and, free radical production (Valdramidis *et al.*, 2010). Sonication has also been reported to inactivate microorganisms and increase antioxidant compounds in chokanan mango juice (Santhirasegaram, Razali and Somasundram, 2013), and in pear juice (Zafra-Rojas *et al.*, 2013), while having

minimal effects on the deterioration of key quality parameters such as colour and ascorbic acid in orange juice during storage (Tiwari *et al.*, 2009). There are very few studies on the effects of US on strawberries/strawberry juice during storage (Tiwari *et al.*, 2009; Tomadoni *et al.*, 2017), and these have concentrated solely on the effect of low frequency US (20 kHz).

Previous work on model enzyme systems of POD (Tsikrika *et al.*, 2018) and PPO (Section 4), showed that high rather than low frequency is more efficient in enzyme inactivation. Specifically, the application of high frequency US at 378 kHz or 583 kHz, was the most effective in the inactivation of these two enzymes. Therefore, the objectives of the present work are:

- To investigate the effect of sonication using 20 kHz (35 W) and two high frequencies at 370 kHz (35 W) and 583 kHz (34 W, and 48 W) on POD and PPO activity as well as in bioactive compounds (anthocyanins, total phenolic content, and antioxidant activity) of strawberry puree.
- To compare the effect of US with that of a thermal control treatment at 40 ^{o}C and of pasteurisation at 90 ^{o}C, on strawberry puree
- To monitor changes on POD and PPO activity and bioactive content of processed and untreated strawberry puree samples during cold storage (4 ^{o}C).

6.2 Materials and methods

6.2.1 **Chemicals**

Potassium phosphate monobasic, potassium phosphate dibasic, pyrogallol, hydrogen peroxide, sodium phosphate monobasic, sodium phosphate dibasic, polyvinylpolypyrrolidone (PVPP), triton X-100, glacial acetic acid, hydrochloric acid, iron (II) sulphate heptahydrate (ferrous sulphate), 2,4,6-Tri(2-pyridyl)-*s*-triazine (TPTZ), iron (III) chloride, Folin & Ciocalteu's phenol reagent, gallic acid, ethanol, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, and (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (Sigma Aldrich Ltd, Gillingham, UK). Catechol, sodium acetate, and sodium carbonate were purchased from Fisher Scientific (Fisher Scientific UK

Ltd, Loughborough). Citric acid was purchased from BDH (BDH Laboratory Supplies, Poole, UK). All chemicals and reagents used were of analytical grade.

6.2.2 **Preparation of strawberry puree**

Strawberries (*Fragaria x ananassa* Duch., cultivar Sonata) were purchased from a local market in Dundee, UK. The fruits were selected, washed thoroughly, drained, destemmed, cut into smaller pieces, and blended using a kitchen blender. The puree was transferred in 50 mL polypropylene sterile containers and then kept frozen at –25 ^{o}C. When required, frozen samples were thawed overnight (12 h) at 4 °C and then diluted with distilled water (1:1 v/v).

6.2.3 **Ultrasonic (US) and thermal treatments**

The US equipment used in these experiments was as described in Section 3.2.3. The high frequency transducer was connected to a glass reaction vessel with a cooling jacket. The starting temperature for all the ultrasonic experiments was 20 ± 2 °C and the temperature profile was recorded every 5 min. It should also be pointed out that the temperature during sonication treatments did not exceed 43 ^{o}C, for all experiments.

6.2.3.1 Sonication with 20 kHz Ultrasound

A standard volume of diluted puree (200 mL, 1:1 v/v dilution) was put into a 400 mL beaker, which was then placed in a 2 L ice-water bath. The 20 kHz sonicator probe was positioned, consistently, 20 mm from the bottom in the 400 mL beaker. A thermometer was also positioned in the beaker to allow the temperature to be monitored. The 20 kHz sonicator experiments were all conducted at 70% amplitude, corresponding at 35 W (determined by calorimetry) and operated on a pulse mode of 4 s on and 2 s off until 60 min of sonication were completed. Volume of 20 mL was removed after 30 and 60 min of sonication, immediately cooled by immersing into an ice-water bath and then subjected to analysis. The rest of the juice, after being cooled, was kept at 4 ^{o}C in order to examine changes during storage.

6.2.3.2 Sonication with 375 and 583 kHz Ultrasound

A standard volume of diluted puree (200 mL, 1:1 v/v dilution) was placed in the reaction vessel, a thermometer was suspended in the reaction liquid and cooling was achieved with a flow of cold water through the reactor jacket. Sonication at 370 kHz US was performed at amplitude corresponding to 35 W, while at 583 kHz US the power levels were 34 W and 48 W. Power levels were determined by calorimetry. Samples (20 mL) were removed after 30 and 60 min of sonication, immediately cooled by immersing in an ice-water bath and then subjected to analysis. The remainder of the puree, after being cooled, was kept at 4 ^{o}C in order to examine changes during storage.

6.2.3.3 Thermal treatment at 40 ^{o}C (control) and pasteurisation at 90 ^{o}C

The effect of heat at 40°C was examined for comparison purposes, considering that the temperature during sonication treatments did not exceed 43 ^{o}C. Thermal treatment at 40 ^{o}C was performed by placing a 250 mL conical flask containing diluted puree (200 mL, 1:1 v/v dilution) in a thermostatic bath, previously equilibrated at 40 ^{o}C. Samples (20 mL) were removed after 30 and 60 min of treatment, immediately cooled by immersing in an ice-water bath and then subjected to analysis. The remainder of the puree, after being cooled, was kept at 4 ^{o}C in order to examine changes during storage

Thermal processing is the most common technique for pasteurisation of fruit juice. Juice pasteurisation aims at a 5 log reduction of the most resistant micro-organisms of public health significance (Barrett, Somogyi and Ramaswamy, 2005). Recent studies on the impact of pasteurisation on the microbial inactivation and phenolic content of fruit juices have used temperatures from 72 to 108 ^{o}C for 1 min or a shorter time period (Chen, Yu and Rupasinghe, 2013). Odriozola-Serrano, Soliva-Fortuny and Martin-Belloso (2008) studied the effect of heat at 90 ^{o}C on strawberry juice and found that there were higher losses on the phenolic content when treatment time exceeded 30 s.

The pasteurisation performed in this study was according to Odriozola-Serrano, Soliva-Fortuny and Martin-Belloso (2008). A 250 mL conical flask containing diluted puree (200 mL, 1:1 v/v dilution) was placed in a thermostatic bath, previously equilibrated at 90 ^{o}C. Samples (20 mL) were removed after 30 and 60 s of treatment, immediately cooled by immersing in an ice-water bath and then subjected to analysis. The remainder of the puree, after being cooled, was kept at 4 ^{o}C in order to examine changes during storage.

The temperature during the treatments was monitored with a laboratory thermometer which was suspended in the juice. The treatment time was calculated when the inner temperature of the juice reached at the desired level.

6.2.4 **Enzyme extraction**

The enzyme extraction of the strawberry puree was performed according to a modified method of Sulaiman *et al.* (2015). Puree (10 mL) was added to a solution containing, 0.2 M sodium phosphate buffer solution (20 mL; pH 7.0) with 4% (w/v) insoluble PVPP and 1% (v/v) triton-X-100, and vortex mixed for 3 min. The homogenate was then centrifuged at 6,000 g for 30 min at 4 ^{o}C. The supernatant containing POD, and PPO was used to assess the activity of these two enzymes.

6.2.5 **POD activity assay**

Peroxidase (POD) activity (ΔA_{420} min^{-1} g^{-1} FW) in strawberry puree was assayed by a modified method of Kwak et al. (1995). The enzyme solution (2.2 mL), potassium phosphate buffer (0.32 mL; 0.1 M, pH 6), pyrogallol (0.32 mL, 5% w/v) and H_2O_2 (0.16 mL; 0.15 M) was added to the sample cuvette. The increase in absorbance at 420 nm was measured by a spectrophotometer (UV-1650 PC, Shimadzu UK Ltd, Milton Keynes) every 20 s for 3 min. The residual activity (RA) of POD was calculated using Eq 6.1:

$$\text{Residual Activity } (\%) = \frac{A_t}{A_0} \times 100$$

Equation 6.1

where A_t and A_0 are, respectively, POD activity after and before the treatment.

6.2.6 **PPO activity assay**

Polyphenol oxidase (PPO) activity (ΔA_{420} min^{-1} g^{-1} FW) in strawberry puree was assayed using the method of Sulaiman *et al.*, (2015), with small modifications. Enzyme solution (300 µL) was mixed with catechol solution (3 mL; 0.07 M in 0.1 M sodium phosphate buffer, pH 6.0). Oxidation of catechol was then measured immediately on a spectrophotometer (UV-1650 PC, Shimadzu UK Ltd, Milton Keynes) at 420 nm and 25 °C every 60s for a total duration of 15 min. The residual activity of PPO was calculated by using Eq 6.1.

6.2.7 **Antioxidant/Phenolic compounds extraction**

The extraction procedure followed the method of Oancea, Stoia, and Coman (2012). Diluted strawberry puree (10 mL, 1:1 v/v dilution) was added to absolute ethanol (20 mL) and then vortexed for 3 min. The homogenate was then centrifuged at 6,000 g for 30 min at 4 ^{o}C, and the supernatant was used for the measurement of the antioxidant activity, total phenolic and total anthocyanin content.

6.2.8 **Anthocyanin Analysis (pH shift method)**

Anthocyanins undergo reversible alterations in their structure with a change in pH exhibited by highly different absorbance spectra. At pH <1.0 anthocyanins are found entirely in their red flavylium (oxonium) form allowing measurement of the total anthocyanins. At pH 3.5 the flavylium is primarily in equilibrium with the colourless carbinol (hemiketal) form, therefore absorbance is due to the polymeric anthocyanins or interfering brown substances (Fig 6.2). The difference in absorbance between pH <1.0 and pH 3.5 is due to the free anthocyanin content.

Figure 6.2 Predominant forms of anthocyanins at different pH values (Cheynier et al., Proceedings ASEV 50th anniversary annual meeting)

The total anthocyanin content (TAC) of strawberry puree was estimated using a pH shift method adapted from Ribéreau-Gayon and Stonestreet (1965).

The strawberry extract (280 µL) was diluted in 3 mL of two different solutions: 2% HCl aqueous solution (pH 0.6) and 0.13 M aqueous buffer solution of sodium carbonate and citric acid (pH 3.5). The absorbance was then recorded at 700 and 520 nm on a spectrophotometer (UV-1650 PC, Shimadzu UK Ltd, Milton Keynes).

TAC is expressed as pelargonidin-3-glucoside (P3g) equivalents, since P3g has been reported as the most common anthocyanin in strawberries (Giusti and Wrolstad, 2001; Aaby *et al.*, 2012; Tonutare, Moor and Szajdak, 2014). The molar coefficient absorptivity (ε; 15600 $M^{-1}cm^{-1}$) (Tonutare, Moor and Szajdak, 2014) and molecular weight (M; 487 g/mol) (Giusti, Rodríguez-Saona and Wrolstad, 1999) were used respectively. The difference in absorbance (ΔA) was calculated according to Giusti and Wrolstad, 2001 as follows:

$$\Delta A = (A_{520}-A_{700})_{pH0.6} - (A_{520}-A_{700})_{pH3.5}$$

Equation 6.2

Anthocyanin content was then calculated as follows:

$$TAC = (\Delta A \times M \times DF \times 1000) / (\varepsilon \times \lambda \times m)$$

Equation 6.3

where DF is the dilution factor, λ is the cuvette optical path length (1 cm) and m is the weight of the sample (g). The total anthocyanin content was expressed as mg P3g per 100 g fresh weight of strawberry puree.

6.2.9 **Total phenolic content (Folin-Ciocalteau method)**

Total phenolic content (TPC) was determined using the Folin-Ciocalteau method as described by Singleton and Rossi (1965). Strawberry extract (0.1 mL) was mixed with Folin-Ciocalteau reagent (5 mL; 0.1 M) and distilled water (0.9 mL). Sodium carbonate (3.5 mL; 1M) was added after 5 min and the mixture was kept in the dark to develop for two hours. The absorbance was then measured at 765 nm and quantification was based on a standard curve prepared with an aqueous gallic acid solution (0.5 – 5.0 mg/mL; $y=0.6719x+0.0463$, $R^2=0.999$, Appendix A, Fig A 9). TPC is expressed as mg equivalents of gallic acid per g fresh weight of strawberry puree.

6.2.10 **Antioxidant Activity (AA)**

The ability of antioxidants to quench free radicals is usually attributed to the primary mechanisms of single electron transfer (SET) and hydrogen atom transfer (HAT) (Prior, Wu and Schaich, 2005). Different antioxidant compounds may act *in vivo* via different mechanisms; thus, a fair estimation of total antioxidant capacity cannot be made, if depended solely on determination of a particular antioxidant compound or just one antioxidant assay. For this reason, Ferric Ion Reducing Antioxidant Power (FRAP) and Trolox Equivalent Antioxidant Capacity (TEAC) assays were employed in this study to evaluate the total antioxidant capacity of strawberry puree.

FRAP assay measures the ability of a solution to reduce ferric-2,4,6-tri-2-pyridyl-s-triazine complex (Fe^{3+}TPTZ) to a blue coloured ferrous complex (Fe^{2+}TPTZ), at low pH, through a SET mechanism, whereas the TEAC assay is based on the measurement of scavenging ability of antioxidants towards long life $ABTS^{\bullet+}$ radicals, via HAT or SET mechanism (Ali, Almagribi and Al-Rashidi, 2016). However, due to the fact that the redox potential of Fe^{3+}TPTZ is comparable with that of $ABTS^{\bullet+}$ (0.68 V), similar compounds can react in both the TEAC and FRAP assays (Prior, Wu and Schaich, 2005).

The FRAP method is simple, fast, inexpensive, with high reproducibility, and correlates well with ascorbic acid and total phenolics, hence it is widely used for determination of antioxidant activity in fruit extract (Prior, Wu and Schaich, 2005; Moharram and Youssef, 2014). TEAC assay is also one of the most commonly used methods for evaluating antioxidant capacity of fruits, vegetables, foods and plants, and is applicable to both lipophilic and hydrophilic antioxidants (Song *et al.*, 2010). It measures the ability of antioxidant molecules to scavenge the blue-green $ABTS^{\bullet+}$ radicals and results are correlated with that of Trolox, a water-soluble vitamin E analogue. Antioxidants react directly with $ABTS^{\bullet+}$, thus causing a reduction to ABTS and a subsequent decolourisation of the solution (Prior, Wu and Schaich, 2005).

6.2.10.1 Ferric Reducing Antioxidant Power (FRAP) assay

FRAP technique measures the ability of antioxidants to reduce ferric iron. The assay was carried out according to Benzie and Strain, (2000). The working FRAP solution was prepared by mixing acetate buffer (100 mL; 0.3 M, pH 3.6) with distilled H_2O (12 mL), $FeCl_2$ (10 mL; 0.01 M) and TPTZ (10 mL; 0.01M in 0.04 M HCl). Strawberry extract (90 µL) was mixed with FRAP reagent (3 mL), the reaction mixture was incubated at 37 ^{o}C for 4 min and then the absorbance was measured at 593 nm on a spectrophotometer (UV-1650 PC, Shimadzu UK Ltd). The absorbance change is due to the combined reductive activity of all the reacting antioxidants present within the sample. Optical density of the sample was compared to a standard ferrous sulphate curve (0-1.0 mM; y=4.3563x + 0.1508, R2=0.998, Appendix A, Fig A 10) and results are expressed as the mean concentration of Fe2+ produced (mM)

6.2.10.2 Trolox equivalent antioxidant capacity assay (TEAC)

Small modifications were made to the method described by Pellegrini et al. (2003). The stock solution of ABTS was produced by mixing an aqueous solution of ABTS (2.5 mL; 7 mM) with aqueous potassium persulfate (44 µL; 140 mM) and keeping the mixture in the dark for 12–16 h before use. At the beginning of the analysis day, an ABTS working solution was obtained by diluting the stock solution to achieve an absorbance of 0.70 – 0.75 at 734 nm, on a spectrophotometer (UV-1650 PC, Shimadzu UK Ltd). Results were expressed as mmol of Trolox equivalents per g fresh weight of strawberry puree, based on a standard curve built with an aqueous Trolox solution (0-250 mmol/L; y=0.3973x + 1.1312, R^2=0.999, y=% inhibition=$\left(1-\frac{\mathrm{As}}{\mathrm{Ab}}\right)*100$, where As is the absorbance of the sample and Ab is the absorbance of the blank solution, Appendix A, Fig A 11).

6.2.11 **Statistical analysis**

All experimental data were analysed using Linear Mixed Model by IBM SPSS Statistics 23. A mixed effect was produced with power (W) as a fixed factor and time (min) and frequency (kHz) as covariates, for every variable studied (POD

and PPO RA, TAC, TPC, and AA as measured by FRAP and ABTS technique). Variation between replicate treatments was treated as a random factor. Main effects of time, frequency, and power on every variable studied and possible interactions between them were also investigated. Normality of residuals was examined with the Shapiro-Wilk's test, while a scatter graph of the residuals and predicted values was plotted in order to check if they are independent. All the plots regarding the assumptions of the tests are in the Appendix A (Fig A 12 – A 28). Differences between means were examined using paired samples t-test. The values were considered significantly different when $p<0.05$. Values presented are the mean of experiments done at least in duplicate and replicated 3 times ($n\geq6$).

6.3 Results and discussion

6.3.1 **Effects of US treatment on enzyme activity**

US at a frequency of 20 kHz (35 W) and two high frequencies at 370 (35 W), and 583 kHz (34 and 48 W) were used in order to examine their influence on the residual activity (RA) of peroxidase (POD) and polyphenoloxidase (PPO) in strawberry puree. Results were also compared with effects of just heat at 40 ^{o}C (control) and 90 ^{o}C (pasteurisation) on POD and PPO RA.

6.3.1.1 Effect on POD activity

In this study it was found that high frequency US treatment at 583 kHz and 48 W was the most effective for POD inactivation (Fig. 6.3). POD RA was 29% and 5% after 30 and 60 min of treatment, respectively. Application of 583 kHz at the lower power of 34 W resulted in 37% (30 min) and 20% (60 min) POD RA, while similar power (35 W) at 20 kHz lead to 47% and 24% POD RA, after 30 and 60 min of treatment, respectively. Less effective was the application of 370 kHz at 35 W, resulting in 63% (30 min) and 35% (60 min). POD RA was 67% and 62%, after 30 and 60 min of control treatment at 40 ^{o}C, respectively, indicating that the low POD activity after US treatment is due to a US rather than heat effect. Pasteurisation at 90 ^{o}C resulted in POD RA of 4% and 2% after 30s and 60s, respectively (data not shown).

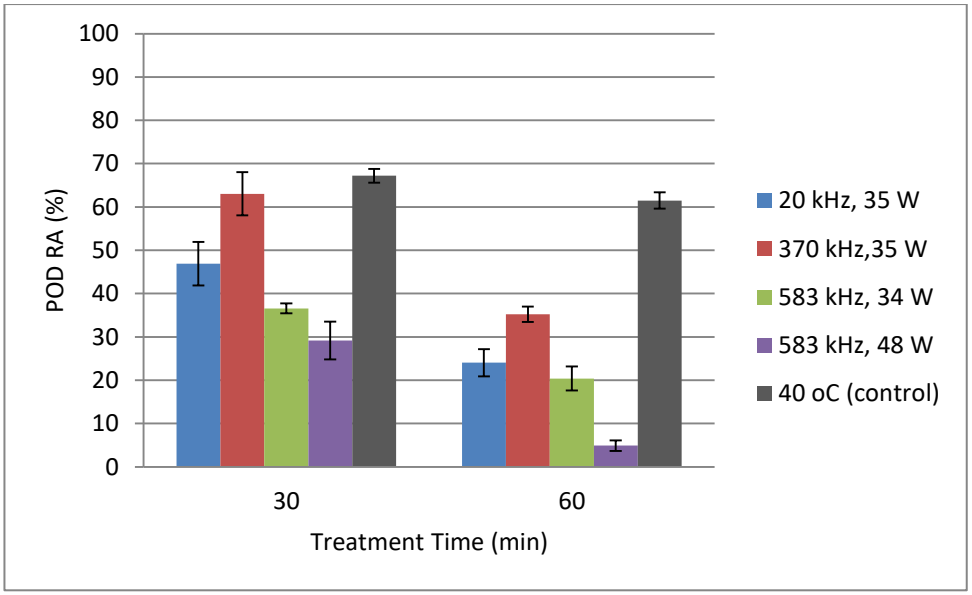


Figure 6.3 Residual Activity (RA %) of POD in strawberry puree treated at low (20 kHz) and high frequency (375 kHz, 583 kHz) at 34-48 W and compared with control at 40 oC. Values presented are the average (n = 6) ±STDEV.

Results of the statistical analysis are shown in Table 6.1. There was a main effect of time ($F(1,68)=580.5$; $p<0.001$) and power ($F(1,68)=14.6$; $p<0.001$) for POD RA. However, frequency did not have a statistical significant effect on POD RA ($F(1,68)=0.5$; $p=0.496$).

Table 6.1 Results of the statistical analysis of POD RA in strawberry juice upon US treatment

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	68	595.835	.000
Time	1	68	565.015	.000
Frequency	1	68	.469	.496
Power	1	68	12.417	.001

a. Dependent Variable: PODRA.

The results obtained in this work of the effect of US on POD RA are in good agreement with a previous study (Tsikrika *et al.*, 2018) in a model system of commercial horseradish peroxidase (HRP). Here, the application of 583 kHz and 48 W in an aqueous solution of HRP for 60 min resulted in complete inactivation of the enzyme, while 20 kHz and 35 W for 60 min lead to 28% HRP RA. On the other hand, the use of 583 kHz at 35 W, and 378 kHz at 32 W was more effective in HRP inactivation (RA about 3% at the end of both treatments) than in peroxidase in strawberry puree. The observed differences can be possibly attributed to the treatment medium, as well as to the extraction source

of peroxidase. In the previous study, peroxidase was in pure form, extracted from horseradish and diluted in water, whereas in the present work the treatment medium and the extraction source was strawberries. It is known that that enzyme inactivation depends very much on the food matrix (Costa *et al.*, 2013). In addition, enzymes are generally more stable in an intact tissue or in a homogenate than in their purified form, because, in real food systems, they are protected by the presence of other materials such as proteins and carbohydrates (Terefe *et al.*, 2010b).

The present work is also in accord with the literature regarding the effects of low frequency US on POD activity. A recent study by Cao *et al.* (2018) showed that when US (20 kHz) with cooling was applied to bayberry juice for 12 min at intensities of 90, 180, 271, 362 and 452 W/cm^2, POD RA was recorded 73 %, 48%, 38%, 19%, and 9%, respectively. Conversely, POD RA after US treatment without cooling, for same duration and intensities 90 – 362 W/cm^2 was 58%, 20%, 8% and 1%, respectively, while complete POD inactivation was achieved at 452 W/cm^2. Ercan and Soysal (2011) observed that POD RA was 64% and 16% after 150s ultrasonic treatment at 23 kHz and powers of 25% and 40%, respectively, while complete inactivation was observed after 150 s and 90 s, at 50% and 75% power respectively. They also reported a possible synergistic effect of ultrasound and heat. Another study on effect of US on pear juice noted that the application of 20 kHz at 750 W for 10 min lead to 43% POD RA at 45 °C, while 10 min sonication at 65 ^{o}C, completely inactivated the enzyme (Saeeduddin *et al.*, 2015). The inactivation times mentioned in these studies are shorter, as compared to the present work; however, it should be pointed out that the US intensities/powers used were much higher, than in this study.

6.3.1.2 Effect on PPO activity

The influence of the same conditions of US treatment on PPO activity in strawberry puree was also evaluated (Fig 6.4). The most effective treatment was 20 kHz at 35 W, resulting in 18% and 9% PPO RA, after 30 and 60 min, respectively. High frequency US at 583 kHz and 48 W lead to 63% and 37% PPO RA after 30 and 60 min of treatment, respectively. PPO RA was recorded 66% and 44% after 30 and 60 min of US treatment at 583 kHz and 34 W,

respectively, while 370 kHz at 35 W resulted in 67% and 48% PPO RA, after 30 and 60 min respectively. Control treatment at 40 ^{o}C, lead to 75% and 67% PPO RA (Fig 6.4), while pasteurisation at 90 ^{o}C after 30 and 60 min caused 8% and 3% PPO RA, respectively.

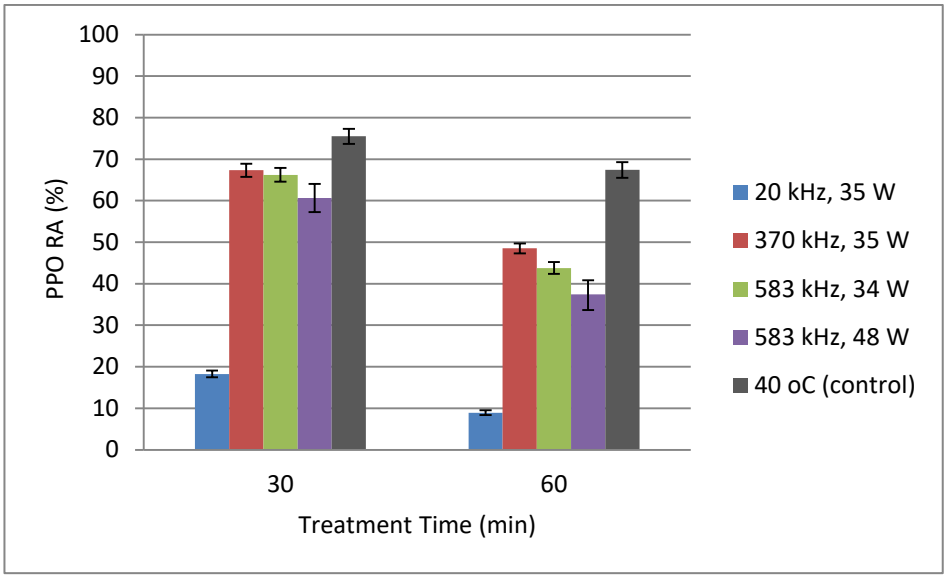


Figure 6.4 Residual Activity (RA %) of PPO in strawberry puree treated at low (20 kHz) and high frequency (375 kHz, 583 kHz) at 34-48 W and compared with control at 40 ^{o}C. Values presented are the average (n = 6) ±STDEV

Results of the statistical analysis are shown in Table 6.2. There was a main effect of time ($F(1,68)=329.5$; $p<0.001$), frequency ($F(1,68)=52.1$, $p<0.001$) and power ($F(1,68)=5.3$; $p=0.024$) for PPO RA but no statistically significant interactions were found.

Table 6.2 Results of the statistical analysis of PPO RA in strawberry juice upon US treatment

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	68	306.528	.000
Time	1	68	329.545	.000
Frequency	1	68	52.130	.000
Power	1	68	5.348	.024

a. Dependent Variable: PPORA.

Previous study with mushroom PPO (see previous section) showed that high rather than low frequency sonication is more effective in PPO inactivation. This is contradicted with the present study, where low frequency US at 20 kHz found to be the most effective. Again, this can be explained by the fact that enzyme

inactivation is very much dependent on the food matrix (Costa *et al.*, 2013). Another possible explanation is that mushrooms contain a latent form of PPO (Şimşek and Yemenicioğlu, 2007), which has not been reported for strawberries (Terefe *et al.*, 2010b). A latent form of PPO could have been released after the application of US in mushroom extract, counteracting the inactivation effect of sonication. Thus, US is found to be less effective in PPO inactivation in mushroom.

Studies on the effect of US on PPO RA have shown a wide variation. Sulaiman *et al.*, (2015) found that RA of strawberry PPO decreased by 75% after 10 min of ultrasound treatment at 24 kHz and 32.5 W. Costa *et al.*, (2011) treated pineapple juice at 19 kHz and intensities of 75, 226, and 376 W/cm^2 for 2-10 min, and observed that PPO activity increased when intensity levels from 150 to 300 (W/cm^2) were applied. The same study showed that PPO RA decreased at higher ultrasound intensity for processing times above 5 min. Similar treatment by Dias *et al.* (2014), i.e. 19 kHz, at 75,118, 224, 330 and 373 W/cm^2 for 2 – 10 min to soursop juice resulted in a slight decrease in PPO RA, independent of the processing time and power intensity used. Abid *et al.* (2014), treated apple juice with ultrasound at 25 kHz, and 70 % amplitude for 60 min and found that there was not any significant decrease in PPO RA. The variation in the published reports suggests that PPO inactivation depends very much on the source and the sub-type of the enzyme (Cheng, Zhang and Adhikari, 2013).

Inactivation of enzymes in foods by US is mainly achieved by physical and/or chemical phenomena such as cavitation, shock waves, and the formation of free radicals by water sonolysis (Raviyan, Zhang and Feng, 2005; Ercan and Soysal, 2011; Sulaiman, *et al.*, 2015a; Cao *et al.*, 2018). As a result, the secondary or tertiary structure of enzymes is modified, leading to loss of their activity. Concomitantly, the free radicals may react amino acids in the enzyme, resulting in alterations to the overall structure and consequently in their catalytic behaviour.

A potential mechanism for the inactivation of POD from ultrasound has been proposed in previous study (Tsikrika *et al.*, 2017) and explained in Section 5. Briefly, UV-visible spectroscopic analysis revealed the removal of the haem

from the active centre of POD, which can possibly explain the inactivation of the enzyme. Additionally, it was speculated that hydrogen peroxide produced by sonication of the water could form hydroxyl radicals by catalytic action of iron in the haem. These radicals could then interact with amino acid residues to form carbon-centred radicals which if present on, for example, Tyr within POD could result in dimerization of these residues resulting in the production of di-tyrosine. Findings of time resolved fluorescence spectroscopy of samples of aqueous solution of horseradish POD previously treated with ultrasound at 378 kHz or 583 kHz (similar conditions with the ones used in this work) revealed the presence of a new fluorescent species within the enzyme, which can possibly be associated with di-tyrosine synthesis.

The inactivation of strawberry POD and PPO by US, as presented in this study, might have been caused by one or a combination of the aforementioned mechanisms. However, the understanding of the specific mechanism(s) by which these enzymes might have been inactivated and how the fruit matrixes influence the efficacy of enzyme inactivation by US, requires further research.

6.3.2 **Effect of US treatment on total anthocyanin content (TAC)**

Anthocyanins have a crucial role in the quality of many fresh and processed fruits and vegetables and they have been associated with health benefits (Giusti and Wrolstad, 2001). Therefore, determination of anthocyanin content is very important in order to assess the quality of raw and/or processed foods.

The effect of different US treatments on TAC of strawberry puree as expressed as pelargonidin-3-glucoside (P3g) equivalents is shown at Fig 6.5. In this current work TAC of untreated strawberry puree was 15.9 mg/ 100 g fresh weight. The anthocyanin content of strawberries has been found to range from 14.8 to 41.8 mg/ 100g (Wrolstad, Putnam and Varseveld, 1970) and as high as 50.3 mg/100 g have also been reported (Pilando, Wrolstad and Heatherbell, 1985). Anthocyanin content can be influenced by numerous factors e.g. genetic (cultivar), the environment, and agronomy as in degree of maturity, edaphic-climatic factors and post-harvest storage (Olsson *et al.*, 2004; da Silva *et al.*, 2007).

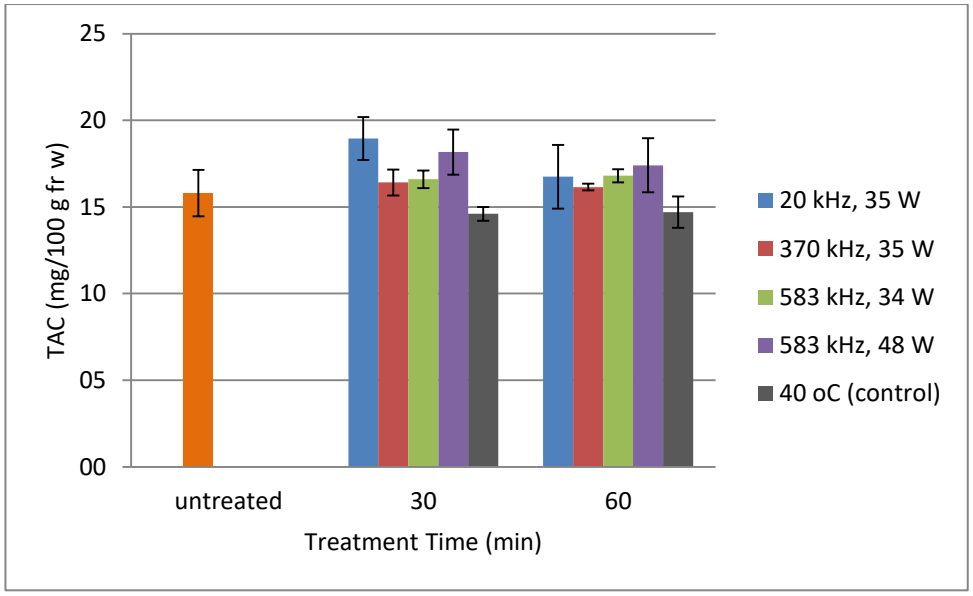


Figure 6.5 Effect of sonication treatments on total anthocyanin content (TAC) as expressed as mg pelargonidin-3-glucoside (P3g) equivalents per 100 g fresh weight of strawberry puree. Values presented are the average (n = 9) ±STDEV.

All US treatments increased the detectable TAC in strawberry puree, compared to those untreated. High frequency at 583 kHz and 48 W, raised TAC 15.2% ($p<0.05$) and 10.1% ($p<0.05$) after 30 and 60 min, respectively. Low frequency US, at 20 kHz and 35 W, increased the TAC in strawberry puree 19.6% ($p<0.05$) after 30 min, whereas, strangely, 60 min resulted in 5.7% ($p<0.05$) increase. The application of high frequency US at 583 kHz and 34 W resulted in 6.3% ($p<0.05$) and 5% ($p<0.05$) increase in TAC of strawberry puree after 30 and 60 min of treatment, respectively, while 370 kHz at 35 W did not had a statistically significant effect. TAC in control samples of strawberry puree was decreased by 7.6% ($p<0.05$) and 7% ($p<0.05$), after 30 and 60 min, respectively, compared to those untreated. Pasteurisation at 90 ^{o}C had a detrimental effect on TAC of strawberry puree resulting in 14.6% ($p<0.05$) and 13.9% ($p<0.05$) decrease after 30 and 60 s, respectively, as compared to untreated samples.

Similar observations were made by Golmohamadi *et al.*, (2013) on raspberry puree. TAC of red raspberry puree was enhanced by 12.6% at 20 kHz and by 6.7% at 490 kHz after 10 min of treatment. No further considerable increase was found after 20 min of sonication at 20 kHz, whereas 986 kHz had no significant effect in TAC content, which they associated with the fact that with the US frequency approaching 1 MHz, the cavitation was not effective in

disrupting the cell walls. Abid *et al.*, (2014b) found no significant changes in TAC of sonicated apple juice samples, after 30 or 60 min treatment at 25 kHz, and 70% amplitude compared to those untreated. Tiwari *et al.*, (2008) treated strawberry juice at 20 kHz and found a slight increase (1 – 2%) in the anthocyanin content of the juice at lower amplitude levels and treatment times, which they attributed to the extraction of bound anthocyanins from the suspended pulp. However, in the same study, they reported a decrease in the anthocyanin content of strawberry juice after US treatment at higher amplitude levels and prolonged period (>5 min), which is in accordance with the present study. Similar observations were made by Tiwari, O'Donnell and Cullen (2009) on blackberry juice after sonication at 20 kHz for 10 min at amplitudes varying from 40% to 100%. The maximum decrease in TAC of blackberry juice was recorded 5%, after US treatment for 10 min at 100% amplitude. They also attributed the loss of anthocyanins, in both strawberry, and blackberry juice, in various factors, such as cavitation, the presence of other compounds (for instance ascorbic acid), and to oxidation reactions, occurring by the interaction of free radicals formed during sonication. Ascorbic acid may interact with the anthocyanins leading to mutual disintegration. Additionally, hydroxyl radicals produced by US are associated with the degradation of anthocyanins because they are responsible for the opening of rings and formation of chalcone (Sadilova, Carle and Stintzing, 2007). The aforementioned factors, and/or a combination of them might have been responsible for the observed decrease in TAC in the present study as well.

Results of the statistical analysis are shown in Table 6.3 and the main effects were time ($F(1,58)=7.6$; $p=0.019$) and frequency ($F(1,41.4)=10.5$, $p=0.011$) but not power ($F(1,27)=3.7$; $p=0.064$) for TAC. No statistically significant interactions were detected. No statistically significant interactions were detected.

Table 6.3 Statistical analysis of TAC in strawberry juice upon US treatment

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	30.165	267.992	.000
Power	1	27.000	3.718	.064
Time	1	59.000	5.796	.019
Frequency	1	27.000	7.556	.011

a. Dependent Variable: TAC.

6.3.3 **Effect of US treatment on total phenolic content (TPC)**

Phenolic compounds are considered as non-essential dietary components in fruits with various activities and chemical structures, and constitute part of the secondary metabolism of plants (Mandave *et al.*, 2014). The effect of different sonication treatments on TPC of strawberry puree, compared with untreated and control samples is shown on fig 6.6. It can be seen that the TPC of untreated strawberry puree was 1.69 ± 0.07 mg/g fresh weight and it is in accordance with values reported in the literature (Medina, 2011; Tulipani *et al.*, 2011; Cassani *et al.*, 2017; Tomadoni *et al.*, 2017). However, the concentration of phenolic compounds in strawberries can be influenced by several factors such as cultivar, ripening stage, growing and other environmental conditions, as well as sample treatment, extraction and quantification methods (Tulipani *et al.*, 2011; Aaby *et al.*, 2012; Velde *et al.*, 2013; Mandave *et al.*, 2014; Teleszko, Nowicka and Wojdyło, 2016; Tomadoni *et al.*, 2017).

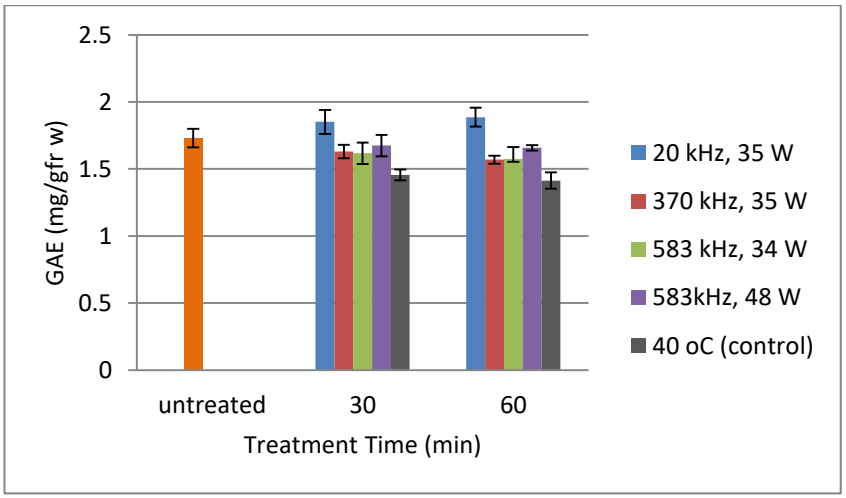


Figure 6.6 Effect of sonication treatments on total phenolic content (TPC) as expressed as gallic acid equivalents (GAE) per g fresh weight of strawberry puree. Values presented are the average (n = 9) ±STDEV.

Low frequency ultrasonic treatment at 20 kHz and 35 W increased the TPC in strawberry puree by approximately 7.2% ($p<0.05$) and 8.9% ($p<0.05$), after 30 and 60 min, respectively, as compared to untreated puree. Higher phenolic levels in various fruit juices, when low US frequencies (20 – 40 kHz) were applied, have been previously reported in many studies; Tomadoni *et al.* 2017 in strawberry juice, Guerrouj *et al.* 2016 in orange juice, Saeeduddin *et al.* 2015 in pear juice, Bhat *et al.* 2011 in lime juice, Abid *et al.* 2014 in apple juice. The increase in TPC in sonicated samples can be possibly ascribed to the enhanced mechanical disruption of the cell walls and the concomitant release of the bound polyphenols via cavitational collapse in the surroundings of colloidal particles (Cheng *et al.*, 2007).

High frequency ultrasonic treatment at 370 kHz and 35 W had as a result 5.9% ($p<0.05$) and 9.3% ($p<0.05$) decrease of TPC in strawberry puree after 30 and 60 min, respectively. Sonication at 583 kHz and 34 W for 30 and 60 min decreased TPC of strawberry puree by about 7.6% ($p<0.05$) and 9% ($p<0.05$), respectively, while 583 kHz and 48 W, did not have any statistically significant effect on TPC of strawberry puree, compared to untreated puree. TPC decreased by 15.9% ($p<0.05$) and 18.4% ($p<0.05$), in control samples after 30 and 60 min of treatment, respectively, as compared to those untreated. As also seen above for the TAC, pasteurisation had a detrimental effect on TPC where heat at 90 ^{o}C for 30 s and 60 s resulted in 11.7% ($p<0.05$) and 17.1% ($p<0.05$) decrease, respectively.

Decrease in TPC of red raspberry purée was detected after sonication for 30 min at 490 kHz by Golmohamadi *et al*. (2013), while Ashokkumar *et al.*, (2008) reported decrease in phenol concentration in a model system when US at 358 kHz and 1062 kHz was used. The latter group correlated the decrease in TPC with an increase in the concentration of hydroxylated products, which could also be the case in the present study.

Changes on TPC are not in agreement with those observed for the TAC, which suggests that anthocyanins may have not contributed much to the phenolic content, of the strawberry puree, as evaluated by Folin-Ciocalteau method, in this study. Strawberries also contain ascorbic acid, which can interfere in the

assay, as it can reduce the Folin-Ciocalteau reagent (Cassani *et al.*, 2018). Therefore, changes on TPC as measured with Folin-Ciocalteau method could be possibly attributed to changes in ascorbic acid levels or other phenolic compounds of the puree, rather than in TAC.

Table 6.4 shows the results of the statistical analysis of TPC in strawberry juice upon US treatment. There was a main effect of time ($F(1,65)=13.4$; $p<0.01$) but not for frequency ($F(1,30)=10.5$, $p=0.174$) nor power ($F(1,30)=3.7$; $p=0.606$) for TPC.

Table 6.4 Results of the statistical analysis of TPC in strawberry juice upon US treatment

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	30.176	64.429	.000
Time	1	65	13.425	.001
Frequency	1	30.000	1.940	.174
Power	1	30.000	.272	.606

a. Dependent Variable: TPC.

6.3.4 **Effect of US on antioxidant activity (AA)**

The antioxidant activity of strawberry puree as evaluated by FRAP analysis is shown in Fig 6.7. High frequency US at 583 kHz and 48 W, for 30 min did not had a statistical significant effect on AA in strawberry puree samples, whereas 60 min of treatment resulted in approximately 2.2% ($p<0.05$) increase, compared to untreated samples. The AA of strawberry puree samples after 30 and 60 min of sonication at 20 kHz and 35 W was approximately 5.9% ($p<0.05$) and 5.4% ($p<0.05$) higher than of those untreated, respectively. High frequency ultrasonic treatment at 370 kHz and 35 W decreased by 5.1% ($p<0.05$) and 5.6% ($p<0.05$) the AA of strawberry puree sample. Use of 583 kHz at 34 W for 30 min did not have a statistical effect on AA of strawberry puree samples, however 60 min resulted in 5% ($p<0.05$) decrease, compared to those untreated. Control samples showed about 9.3% ($p<0.05$) and 11.7% ($p<0.05$) decrease in strawberry puree AA after 30 and 60 min of treatment, respectively. Pasteurisation decreased AA about 8% ($p<0.05$) after 30 s, and 9.5% ($p<0.05$) after 60 s, as compared to untreated puree.

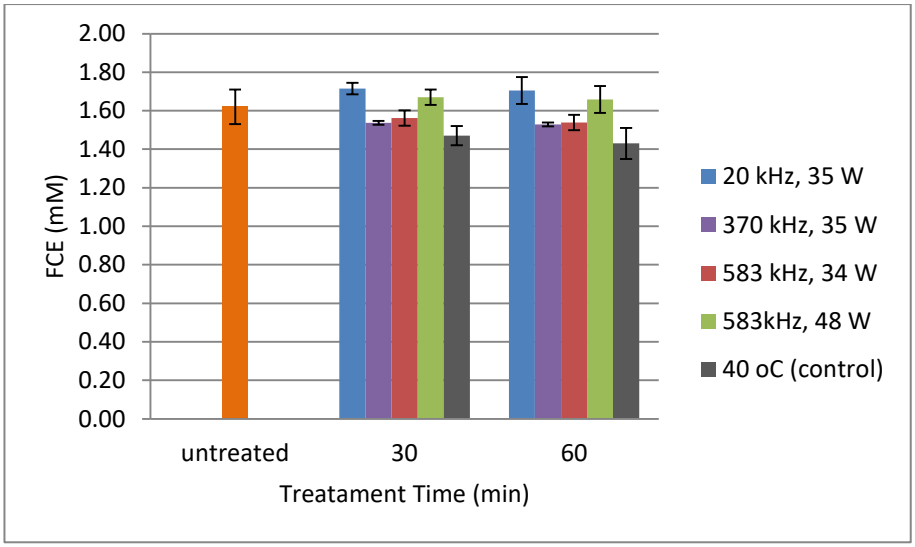


Figure 6.7 Effect of sonication treatments on antioxidant activity of strawberry puree as expressed as mM ferric chloride equivalents (FCE). Values presented are the average (n = 9) ±STDEV.

The results of the statistical analysis are shown in Table 6.5. There were main effects of frequency ($F(1,30)=16.5$; $p<0.001$ and power ($F(1,30)=5.2$; $p=0.030$), but not of time ($F(1,65)=13.4$; $p=0.458$) for AA as measured by FRAP assay.

Table 6.5 Results of the statistical analysis of AA as measured by FRAP assay in strawberry juice upon US treatment

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	30.008	837.356	.000
Time	1	65.000	33.635	.000
Frequency	1	30.000	.015	.902
Power	1	30.000	1.240	.274

a. Dependent Variable: ABTS.

Changes in AA as estimated by FRAP analysis are similar to those observed in TPC for most samples. The explanation of the increasing trends might be the addition of generated sonochemically hydroxyl radicals ($^{\bullet}OH$) to the aromatic ring of phenolic compounds, in ortho-, meta-, or para- position. It is suggested by Ashokkumar *et al.* (2008) that the hydroxylation in ortho- or para- position in phenols increases their antioxidant activity, which is in accordance with the present study. Similarly, Yeoh and Ali (2016) reported a significant increase in total antioxidant capacity of fresh-cut pineapple, as determined by FRAP, in sonicated samples at 37 kHz and 25 W or 29 W for 10 to 15 min, as compared to the controls. They also reported a significant correlation between the total

antioxidant activity as determined by the FRAP technique and total phenolic content. Phenolic compounds are generally considered bioactive as antioxidants because of their capacity to chelate metals, inhibit lipoxygenase and scavenge free radicals (Mandave *et al.*, 2014).

The sonicated samples at 583 kHz and 48W, exhibited higher AA but had lower TPC, as compared to those untreated. However, TAC in these samples was higher compared to those untreated, which might explain the increase in their overall antioxidant activity. A higher reducing power (Fe^{3+}–Fe^{2+} transformation) in sonicated, at 40 kHz and 130 W, chokanan mango juice was also observed by Santhirasegaram *et al.* (2013). The increase in the antioxidant activity by ultrasound might be possibly attributed to the effective removal of occluded oxygen (Knorr *et al.*, 2004).

Fig. 6.8 shows the effect of ultrasound on the total antioxidant activity of strawberry puree as evaluated by the TEAC analysis. All ultrasonic and both thermal (at 40 ^{o}C and 90 ^{o}C) treatments caused a slight decrease, <1% ($p<0.05$) in the AA of strawberry puree, compared to untreated puree.

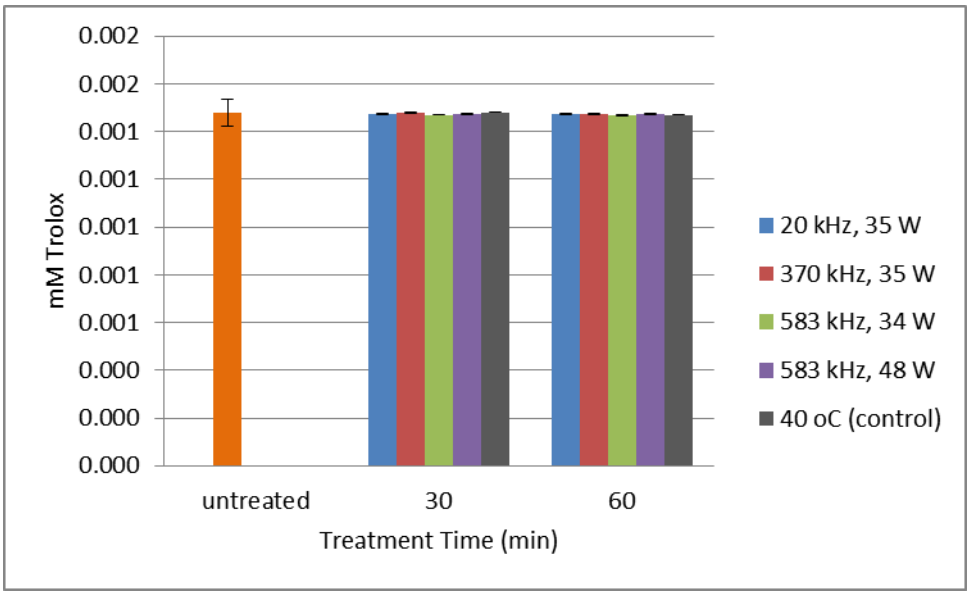


Figure 6.8 Effect of sonication treatments on antioxidant activity of strawberry puree as expressed as mM Trolox equivalents. Values presented are the average (n = 9) ±STDEV.

Concerning statistical analysis, similar findings were observed for AA determined by TEAC technique (Table 6.6). Time had a statistically significant

effect (F(1,65)=33.6; p<0.001, however frequency and power did not (F(1,30)=0.1; p=0.759, F(1,30)=1.8; p=0.184, respectively).

Table 6.6 Results of the statistical analysis of AA as measured by TEAC analysis in strawberry juice upon US treatments

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	30.008	837.356	.000
Time	1	65.000	33.635	.000
Frequency	1	30.000	.015	.902
Power	1	30.000	1.240	.274

a. Dependent Variable: ABTS.

Similar results have been observed by Zafra-Rojas *et al.* (2013) who reported that the antioxidant activity of purple cactus pear fruits after sonication at 60% and 80% amplitude levels for 15 min did not change. Golmohamadi *et al.* (2013) found that the antioxidant activity of red raspberry puree as determined by DPPH radical scavenging assay, did not change significantly (p<0.05) after the application of high intensity (20 kHz) or medium intensity US (490 kHz). On the other hand, Santhirasegaram, Razali and Somasundram (2013) reported a slight increase in antioxidant activity, as determined by DPPH assay of Chokanan mango juice sonicated at 40 kHz and 130 W for 30 and 60 min, which is in agreement with the present work, since DPPH and TEAC have similar mechanism or reaction (Yeoh and Ali, 2016).

A possible explanation of the contradicting results between FRAP and TEAC analysis could be that antioxidant capacity was measured using different methods. Antioxidant activity might be due to a combined effect of different compounds, acting either synergistically or antagonistically. Furthermore, the antioxidant activity of phenolic compounds might be influenced by various factors such as the oxidation system, degree of glycosylation, partition coefficient, concentration, and parameter measured (Hassimotto *et al.* 2005). In fact, flavonoids might exert antioxidant or pro-oxidant activities, depending on the radical-generating system (Cao, Sofic and Prior, 1997).This may explain why despite the high anthocyanin levels observed in the sonicated strawberry puree samples in this study, AA as measured by TEAC exhibited decreasing

trends. Pro-oxidant activity has also been reported for ascorbic acid in strawberry (Kalt *et al.*, 1999), which might have also contributed in the aforementioned findings of this study.

6.3.5 **Changes during storage**

The effect of US treatments on POD and PPO RA, TAC, TPC, and AA as estimated by FRAP and TEAC analysis in strawberry puree was evaluated after 24, 72 and 120 h of cold storage at 4 ^{o}C, in the dark. Any changes during storage were also determined in the control (40 ^{o}C) and pasteurised at 90 ^{o}C samples and in those untreated, for comparison purposes. According to the literature (Tomadoni *et al.*, 2017), unprocessed strawberry juice showed the presence of 7 log CFU/mL of microorganisms after 5 days of cold storage (5 ^{o}C), therefore the max storage time for this study was set at 5 days.

Overall, sonicated samples retained significantly lower POD and PPO RA than of those untreated or the controls. Anthocyanin content, total phenolic content, and antioxidant activity as estimated by FRAP and TEAC analysis, gradually decreased with the increase of storage time in most strawberry puree samples. It should be also noted that levels of these bioactives were higher in samples subjected to US than in the controls, and those pasteurised.

Figure 6.9 (a) and (b) shows POD and PPO RA in all the strawberry puree samples during storage at 4 ^{o}C. There is a decreasing trend after 5 days of storage in all samples. No reactivation was observed in the sonicated samples treated at 583 kHz and 48 W, and 20 kHz and 35 W, regarding POD and PPO respectively. Additionally, all sonicated samples had lower POD and PPO RA than of those untreated, and control ones. Also, no reactivation for POD and PPO occurred in the pasteurised samples at 90 ^{o}C. All results were statistically significant ($p<0.05$).

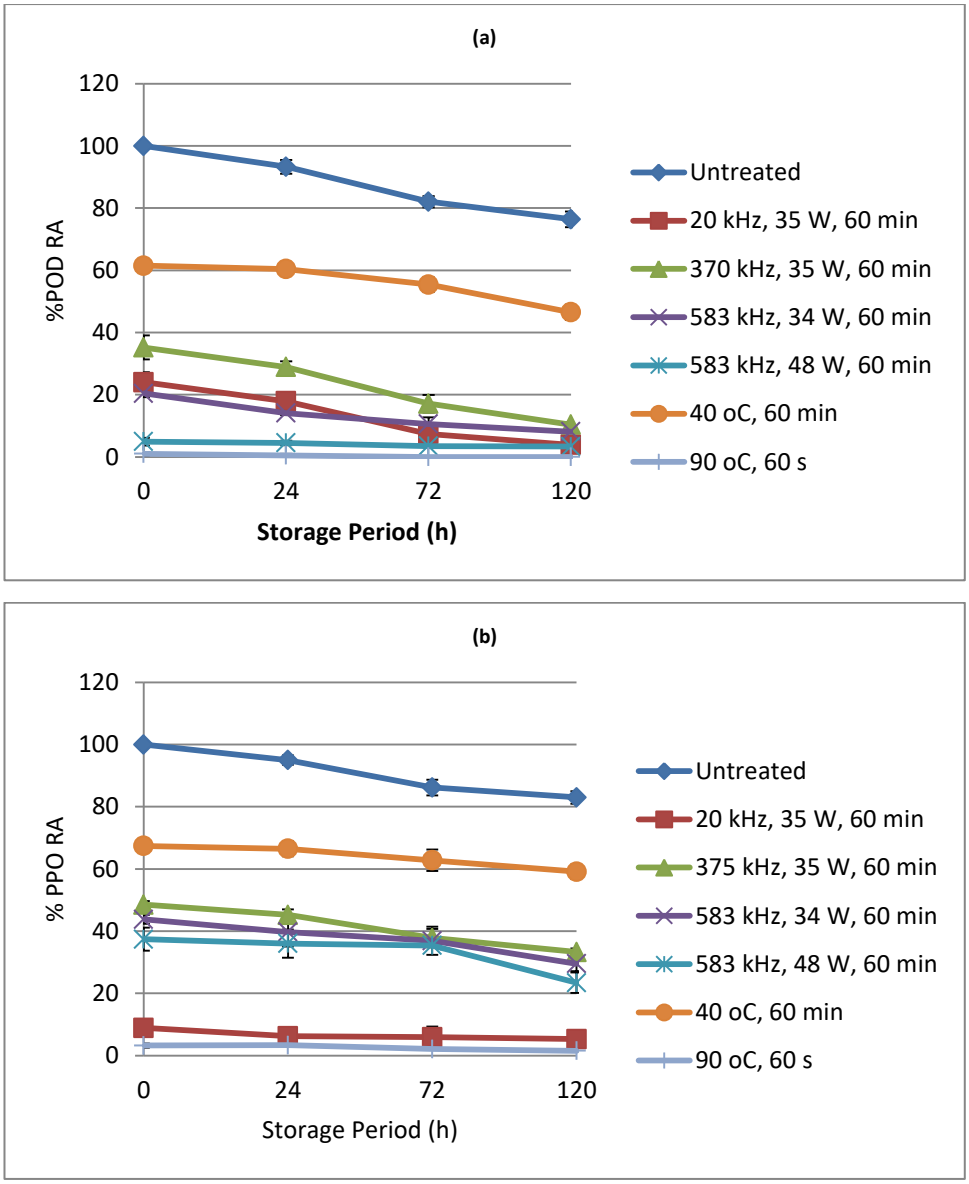


Figure 6.9 Residual activity (RA%) of POD (a) and PPO (b) of unprocessed, sonicated and thermally treated strawberry puree during storage. Values presented are the average (n=6) ± STDEV.

Total anthocyanin content gradually decreased with the increase of storage time in all strawberry puree samples (Fig 6.10). Strawberry puree samples treated at 583 kHz and 34 W had no statistical significant changes ($p>0.05$) in TAC after 72 h of storage however, all other sonicated and untreated samples had statistically significant decrease ($p<0.05$) during storage. Control samples exhibited statistically significant changes ($p<0.05$) in TAC during storage, except for the samples examined after 24 h of storage. All pasteurised samples at 90 ^{o}C showed statistically significant ($p<0.05$) decrease in TAC during storage.

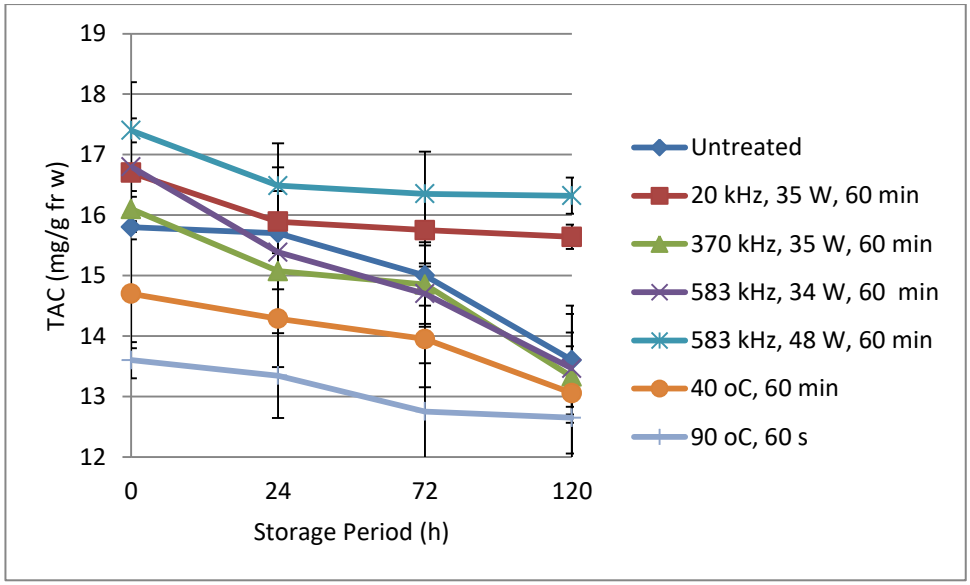


Figure 6.10 Total anthocyanin content (TAC) in strawberry puree during storage (4°C) as expressed as mg pelargonidin-3-glucoside (P3g) equivalents per 100 g fresh weight of blended strawberries during .Values presented are the average (n=9) ±STDEV.

Similar findings regarding TAC of strawberries during cold storage has also been reported by Octavia and Choo (2017). Tiwari et al. 2008 observed losses in anthocyanin content, and a simultaneous degradation of ascorbic acid, in sonicated strawberry juice during storage, indicating an interaction between these two compounds. Cao et al. (2012b) also ascribed anthocyanin decay to oxidative degradation of ascorbic acid. It is hypothesised that the formation of hydrogen peroxide which follows the oxidation of ascorbic acid, causes a nucleophilic attack of the C2 of anthocyanins, and consequently their degradation (García-Viguera and Bridle, 1999). Numerous factors can also affect anthocyanins' stability in fruits, vegetables as well as in their products, including oxygen, enzymes, metal ions and sugars (Patras *et al.*, 2010). These factors may have had an effect on TAC of strawberry puree samples during storage in the present work as well.

Figure 6.11 shows TPC in all strawberry puree samples during storage. Samples treated at 583 kHz and 48 W did not exhibit any statistically significant ($p>0.05$) changes in TPC during storage. TPC in samples treated at 20 kHz and 35 W did not show statistically significant changes after 24 h of storage however, after 72h and by the end of the storage period decreased statistically significant ($p<0.05$). There was a slight increase, but statistically significant ($p<0.05$) in TPC of samples sonicated at 370 kHz and 35 W after 72 h of

storage and for those treated at 583 kHz 34 W, after 120 h of storage. Control samples had a statistically significant ($p<0.05$) decrease in TPC after 24 h of storage, but for the rest of the storage period the changes were not statistically significant ($p>0.05$). The TPC in pasteurised samples showed a statistically significant decrease ($p<0.05$) throughout storage.

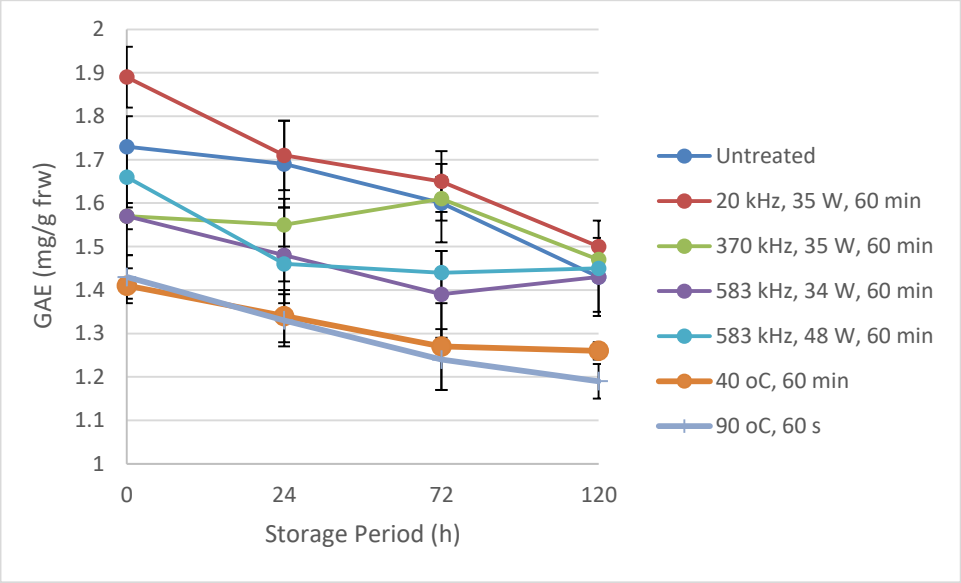
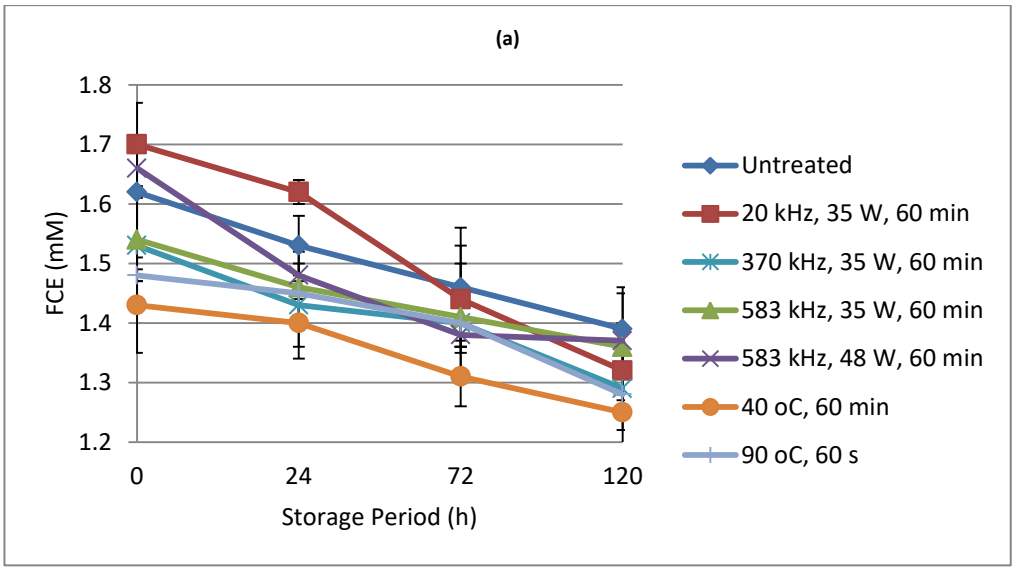


Figure 6.11 Total phenolic content (TPC) as expressed as gallic acid equivalents (GAE) per g fresh weight of strawberry puree during storage. Values presented are the average (n=9) ±STDEV.

Tomadoni *et al.* (2017) observed a decrease in TPC in all strawberry juice samples, sonicated or controls, after 3rd day of storage, followed by an increase until the 10th day. They attributed the overall increase to cellular senescence, which might have released phenolic and amino acids. Peroxidase and polyphenoloxidase activity has been associated with phenolic degradation in processed strawberries (López-Serrano and Ros Barceló, 2002; Chisari, Barbagallo and Spagna, 2007). In the present study, POD and PPO residual activities decreased significantly with the advance of storage time. However, both enzymes retained some residual activity, thus the observed decrease in TPC of strawberry puree samples during storage, could be possibly attributed to POD and PPO RA. Losses in TPC of sonicated fresh-cut pineapple after five days of storage have been reported by Yeoh and Ali (2016), who also found that there was an inverse correlation between the power input of US and the phenolic levels. Moreover, they suggested that TPC in fresh-cut pineapples was positively influenced by phenylalanine ammonia lyase (PAL) activity, a phenolic

metabolism enzyme, which is activated after mechanical operations in fruits and vegetables. A developmental-dependent expression of PAL activity and accumulation of phenolic substances has also been observed in strawberries (Cheng and Breen, 1991). Hence, the recorded decrease in TPC during storage in the present study might have been influenced by low PAL activity. Cao *et al.* (2012b) ascribed TPC decrease during storage in clear and cloudy strawberry juices, previously processed by high hydrostatic pressure, to oxidation degradation of phenolic compounds and their polymerisation with proteins. The oxidation of phenolics could possibly explain the TPC degradation during storage, as recorded in this study as well.

There was a decreasing trend with the increase of storage time in antioxidant activity of strawberry puree samples as measured by both FRAP and TEAC assays (Fig 6.12).



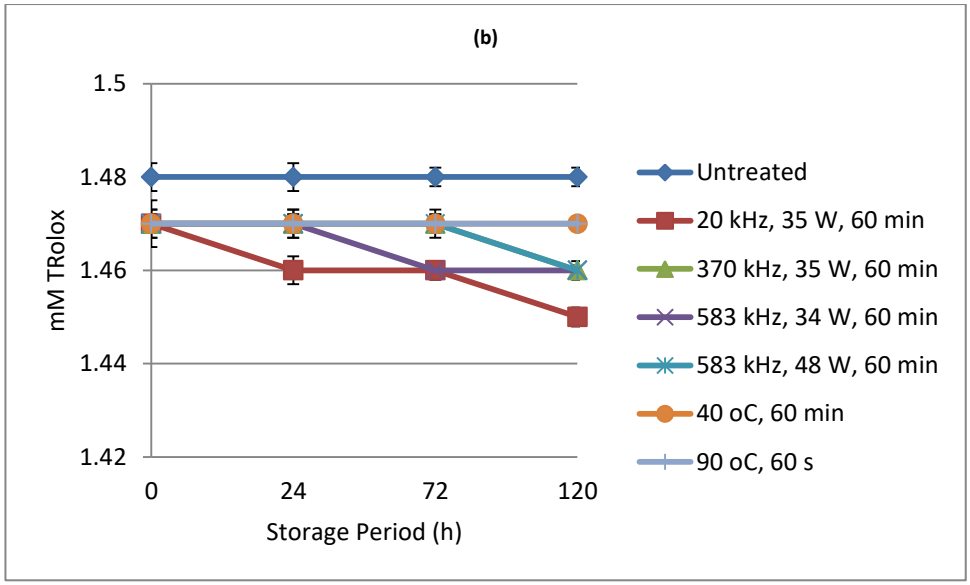


Figure 6.12 Antioxidant activity of all strawberry puree samples as measured by FRAP (a) and TEAC (b) assay during storage. Values presented are the average (n=9) ± STDEV.

Untreated samples did not have any statistically significant ($p>0.05$) changes in AA as measured by the FRAP assay after 24 h of storage, but for the rest of the storage time AA there was a statistically significantly decrease ($p<0.05$). Similar results were recorded for the AA of samples treated at 583 kHz and 48 W, whereas for the remainder of the sonicated samples AA showed a statistically significant ($p<0.05$) decrease throughout storage. Control samples had no statistically significant changes ($p>0.05$) in AA as determined by the FRAP assay after 24h of storage, but AA dropped significantly ($p<0.05$) after 72 h and 120 h of storage. AA in pasteurised samples decreased significantly ($p<0.05$) during storage as well.

Similarly, with the findings of the FRAP assay, AA in untreated strawberry puree samples as determined by the TEAC technique, did not change significantly ($p>0.05$) by the end of the storage time. AA in sonicated samples at 583 kHz and 48 W, 370 kHz, and 20 kHz at 35 W did not change significantly ($p>0.05$) after 24 h of storage but decreased significantly for the rest of the storage period. Conversely, AA in samples treated at 583 kHz and 34 W decreased significantly ($p<0.05$) after 24h and 72 h of storage, but did not change significantly after 120 h. Control, and pasteurised samples showed no statistically significant ($p>0.05$) changes in AA, as measured by the TEAC assay.

Decrease in antioxidant activity of sonicated fresh-cut pineapple, as measured by the FRAP and ABTS analysis, during storage, was also reported by Yeoh and Ali (2016). In addition, they found a significant correlation between total phenolic content and antioxidant activity, suggesting that the latter was mainly affected by the concentration of polyphenols. This could also be the case in this current study, since TPC and FRAP antioxidant activity followed a similar trend during storage. Khandpur and Gogate (2015) also attributed the decrease in antioxidant activity against DPPH radicals, after 10 weeks of storage, to losses in phenolic and ascorbic acid content of fruit and vegetable juices (orange, sweet lime, carrot and spinach) treated with ultrasound and ultraviolet irradiation.

6.4 Conclusion

The present study has examined the influence of US at low (20 kHz) and high frequency (370 kHz, and 583 kHz) on POD and PPO activity and bioactive compounds of strawberry puree after treatment and during storage. A control treatment at 40^{o}C (maximum temperature reached during sonication) and pasteurisation at 90 ^{o}C were also performed for comparison purposes. Results showed that POD and PPO activity was significantly lower in sonicated samples than in the control or those untreated throughout the storage period. Furthermore, sonication enhanced anthocyanin content; total phenolic content and antioxidant activity, as determined by FRAP analysis, in strawberry puree samples, as compared to control, pasteurised, and those untreated. A slight decrease in antioxidant activity, as determined by TEAC analysis was observed in all treated samples, as compared to those untreated. Pasteurisation was more effective in POD and PPO inactivation in strawberry puree than US treatments. However, pasteurisation had a detrimental effect on the bioactive content of strawberry puree samples. In addition, the bioactive content in all sonicated samples was higher than in the controls or those pasteurised, during storage. These findings indicate that ultrasound could be an alternative to conventional thermal processing of strawberry puree avoiding quality losses of the product and at the same time effective against enzymes responsible for food deterioration. Further research is required in order to evaluate ultrasound

treatment in combination with different non-thermal or mild temperature thermal technologies with regard to shelf-life extension of the product, while maintaining its physicochemical, organoleptic and nutritional properties.

7 General Conclusions and Future Work

The present thesis examines ultrasound (US) technology as an alternative to traditional methods of food processing. For this purpose, the effect of US on the catalytic activity of food enzymes in pure solution and a juice system was investigated. Structural changes in these enzymes after US treatment were studied to produce possible mechanisms of enzyme inactivation upon US treatment. The impact of US on the changes in antioxidant activity, polyphenols, and anthocyanins before/after US treatment in comparison with untreated and thermally processed juice samples was also evaluated.

Section 3 of this thesis concerns the effect of different US frequencies and power levels on the catalytic activity of a commercial horseradish peroxidase (HRP). High US frequencies were found to be more effective in HRP inactivation than low frequency at similar acoustic power levels. HRP residual activity (RA) upon sonication at 378 kHz (32 W) and 583 kHz (34 W) was found to be 10% after 45 min of treatment, whereas use of 20 kHz (35 W) for 60 min resulted in 28% HRP RA. Complete inactivation of HRP occurred after 30 min of US treatment at 378 or 583 kHz at 48 W, while inactivation kinetics followed a first order model after every US treatment. A control thermal treatment was also performed, and this had a limited effect on HRP activity, indicating that HRP inactivation upon sonication was due to the effect of US rather heat. However, due to the prolonged treatment time the use of high frequency US alone would not be suitable for the food industry.

Similar findings were observed at the Section 4, which focused on the effect of US on polyphenoloxidase (PPO). The impact of different US frequencies, powers and times on the activity of mushroom PPO was investigated. Again, high US frequencies were more effective than low frequency. The application of 378 kHz (32 W) and 583 kHz (34 W) in mushroom PPO lead to a similar RA after 90 min of treatment (3%, 2%, respectively) whereas the use of 20 kHz (35 W), for the same period, resulted in 59% PPO RA. Complete inactivation of mushroom PPO occurred after 60 min of US treatment at 378 or 583 kHz at 48 W. In fact, mushroom PPO under the conditions studied was more resistant to US treatment than HRP, indicating that inactivation by US depends very much

on the type and source of the enzyme. Inactivation kinetics of PPO residual activity after high frequency sonication fitted the Weibull model well, whereas the kinetics data from 20 kHz treatment followed a first order model. These findings also suggest that enzymes can exhibit different behaviours with regards to the inactivation method applied.

Structural changes of HRP and PPO upon US and thermal treatment were studied using Time Resolved Fluorescence Spectroscopy in Section 5, in order to gain an insight into possible inactivation mechanisms. Analysis revealed that for HRP the mechanism includes haem elimination and alterations to the secondary structure. The formation of hydrogen peroxide from water caused by cavitation induced by US interacts with the iron present in the haem, leading to the formation of a di-tyrosine structure in the treated HRP. Concerning PPO, fluorescence anisotropy indicated alterations in the tetramer structure occurred however, further studies are required in order to delineate the mechanism of these changes.

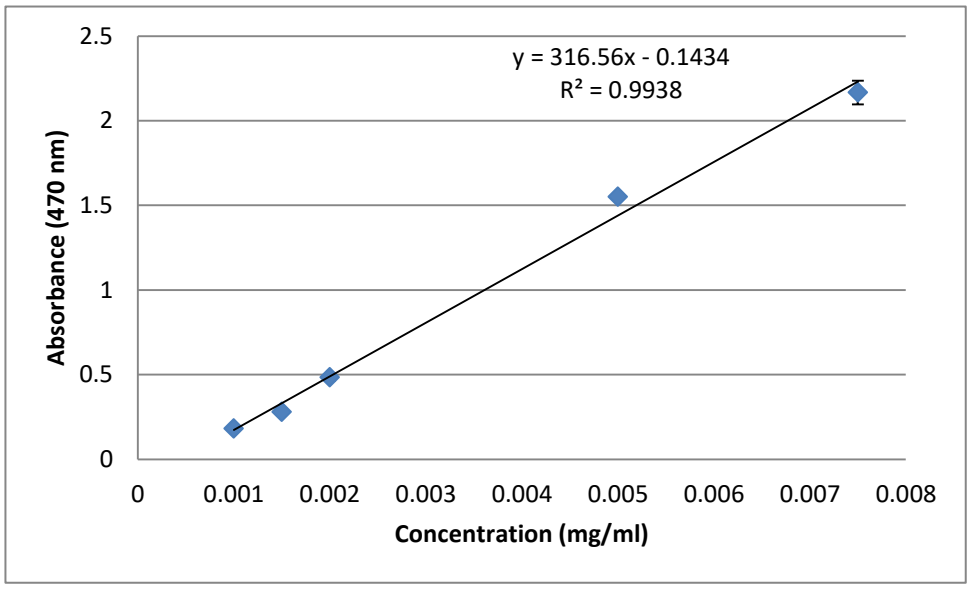
Section 6 of the thesis examines the inactivation of POD and PPO by US and discusses the effects of sonication on bioactive compounds in strawberry puree after treatment and during storage. For comparison purposes a control thermal treatment (40 ^{o}C) and pasteurisation (90 ^{o}C) were also performed. The results show that POD and PPO RA was significantly lower in sonicated strawberry puree samples compared to those untreated and in the controls, after treatment and throughout the storage period. High frequency US at 583 kHz (48 W) lead to 5% strawberry POD RA after 60 min of treatment, whereas POD RA was 62% in the control strawberry puree samples. US at 20 kHz (35 W) for 60 min resulted in 9% strawberry PPO RA, whereas control treatment lead to 67% PPO RA. POD and PPO activity strawberry puree upon US treatment was different compared to the model systems (HRP and mushroom PPO) supporting the previous observations regarding different sub-types of enzymes and indicating that the complexity of the food matrix greatly affects US inactivation efficiency. Pasteurisation inactivated completely both enzymes, however had a detrimental effect on anthocyanins, total phenolic content and total antioxidant activity of strawberry puree (13.9%, 17.1%, and 9.5 % decrease after 60 s of treatment,

respectively). On the contrary, after treatment and during storage total anthocyanin and phenolic content as well as antioxidant activity, in sonicated strawberry puree samples were enhanced as compared to those untreated and the controls.

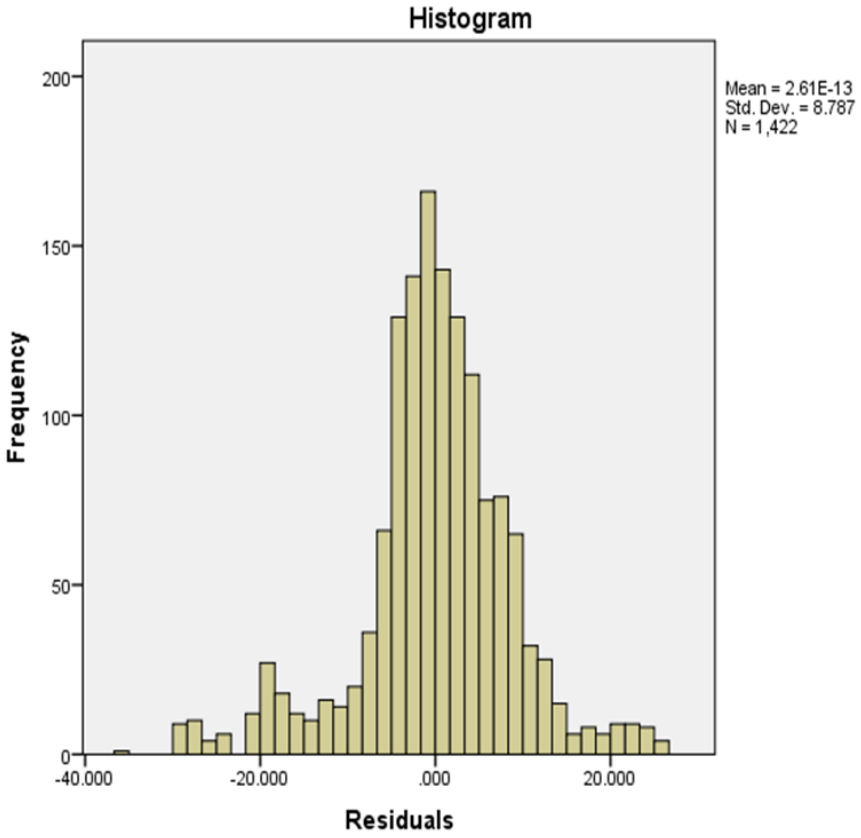
This study provides a scientific and technological basis to further develop high frequency sonication as an alternative to conventional thermal processing of strawberry puree. High frequency US was found to be effective against enzymes responsible for food deterioration and at the same time quality losses of the product were avoided.

Possible future work could investigate the effect of high frequency US in combination with other methods and/or the addition of other components in strawberry puree in order to increase the rate of enzyme inactivation and subsequently decrease the processing time. Microbial inactivation as well as of other enzymes responsible for food deterioration (e.g. lipoxygenase, and pectinmethylesterase) in strawberry puree and other fruits and vegetables by US treatment could also be examined in order to get a better understanding regarding the safety of the product upon US treatment. The bio-accessibility of certain bioactives in strawberry puree as well sensory evaluation of strawberry puree upon high frequency sonication could provide additional information of the impact of high frequency US on the quality of strawberry puree. Additionally, mathematical modelling and process optimisation could contribute towards the scaling-up of US processing.

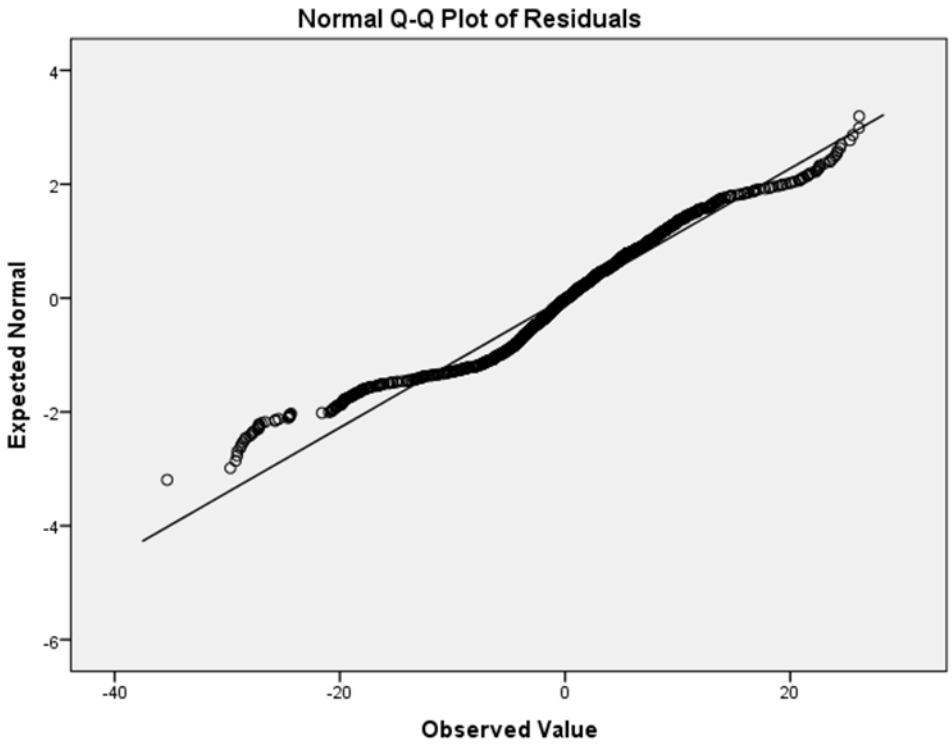
8 Appendix A



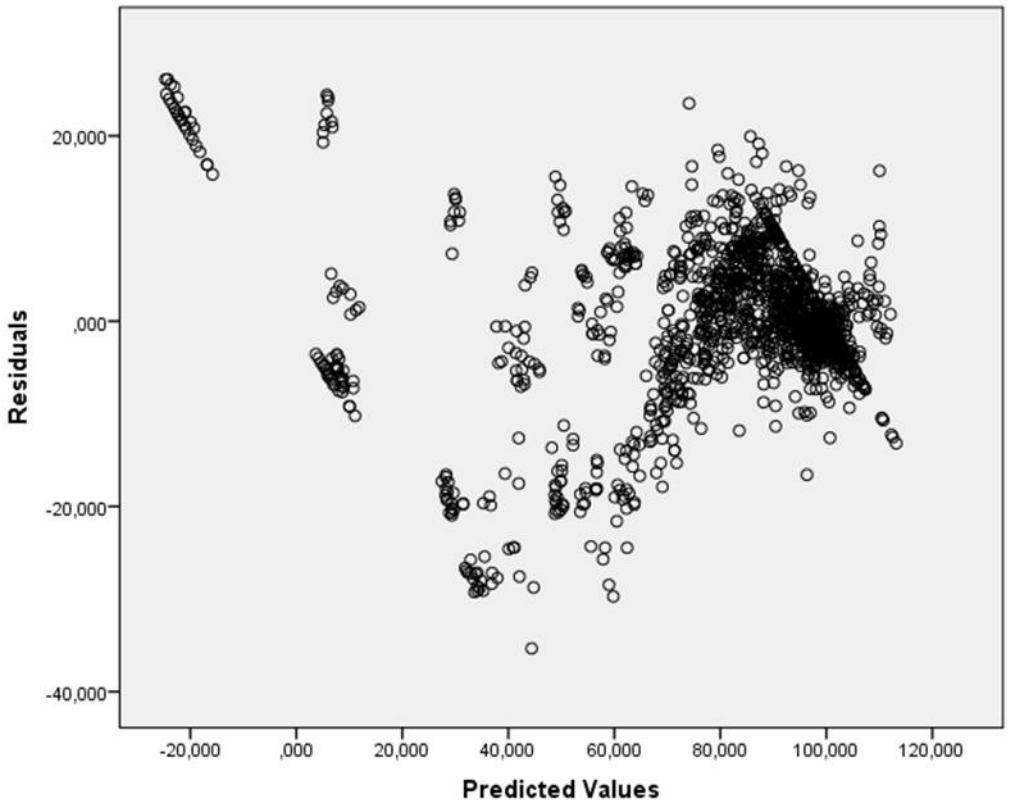
A 1 Graph of the absorbance of different HRP solutions



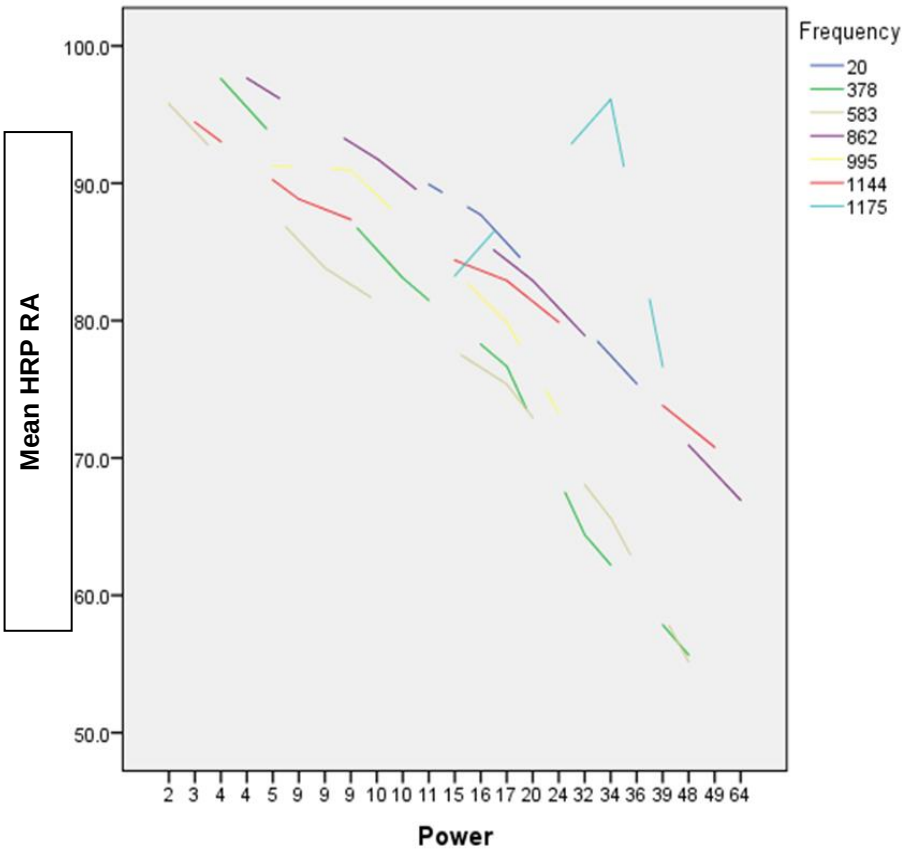
A 2 Normal distribution histogram of HRP RA residuals



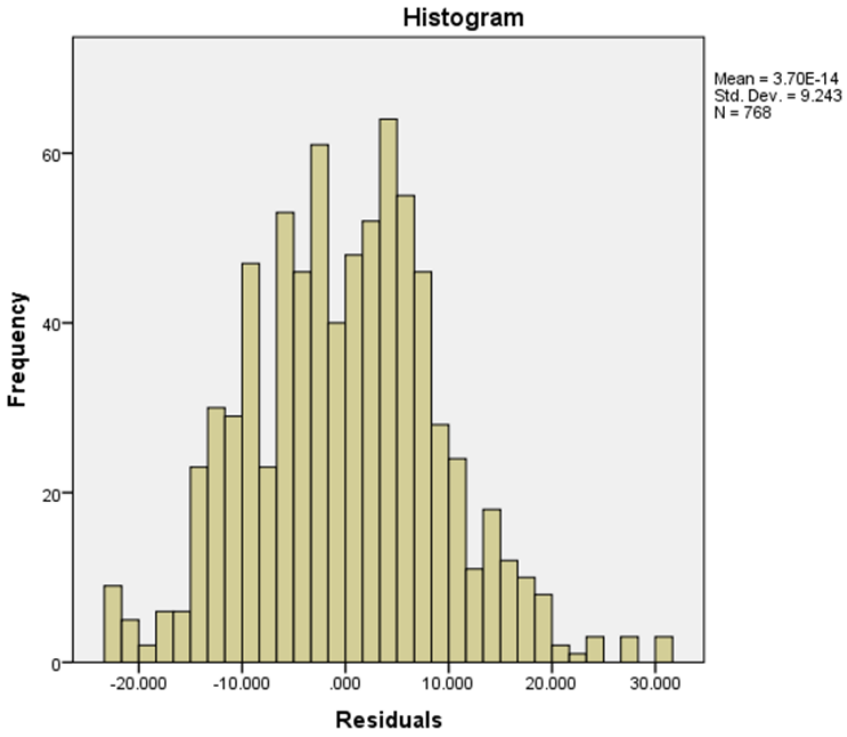
A 3 Graph of linearity of residuals (predicted values versus observed values) for HRP RA



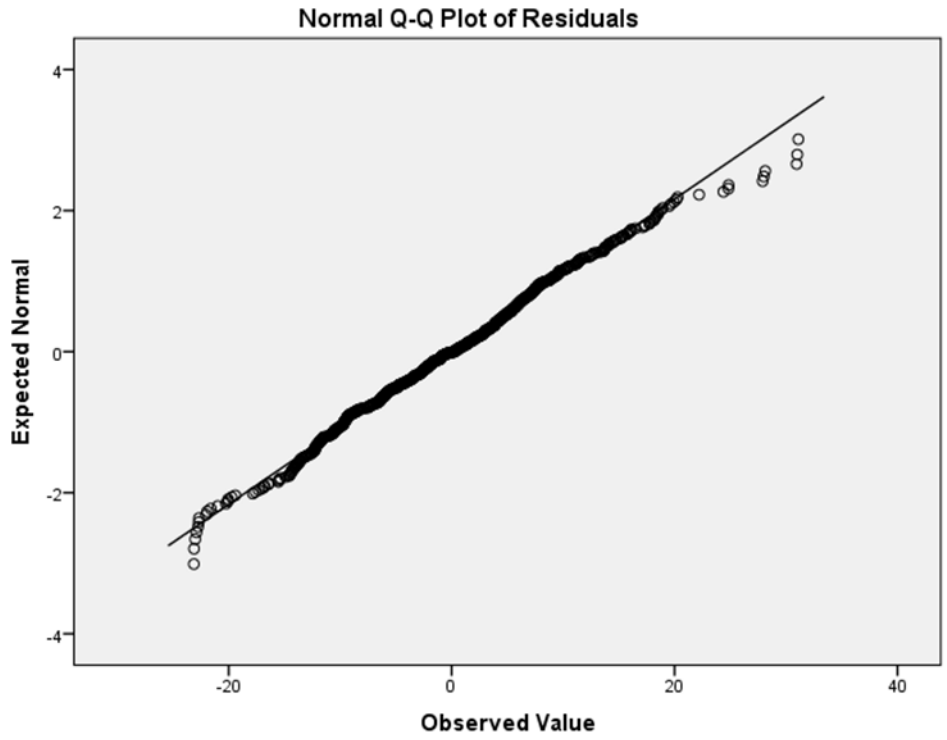
A 4 Graph of independence of residuals (residuals versus predicted values) of HRP RA



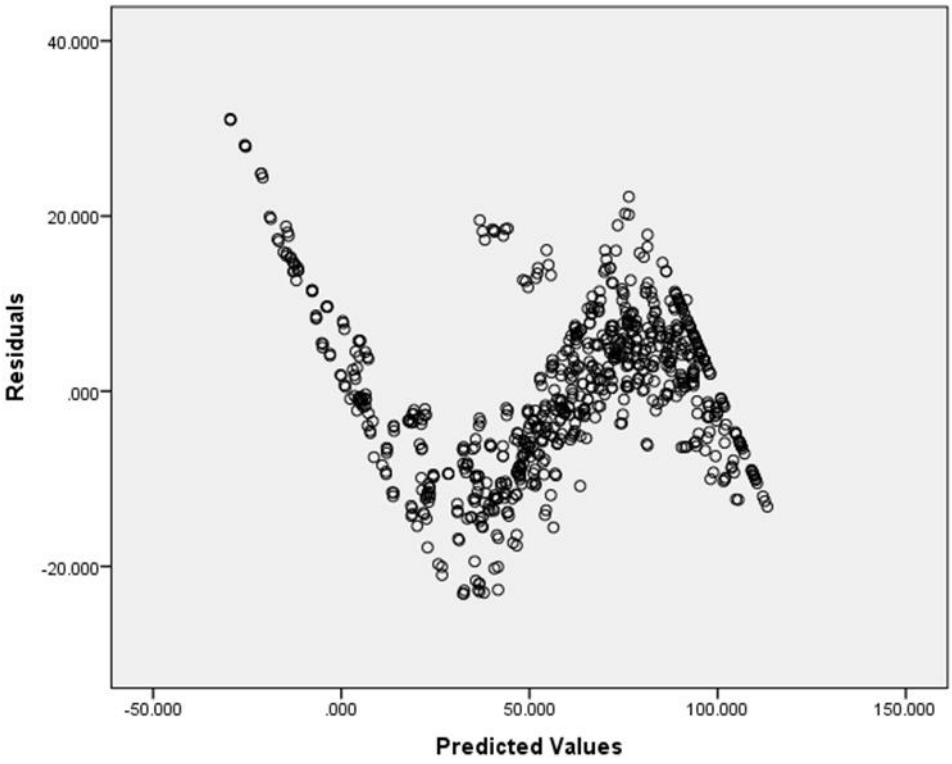
A 5 Graph of HRP RA versus power (W) at the different frequencies studied



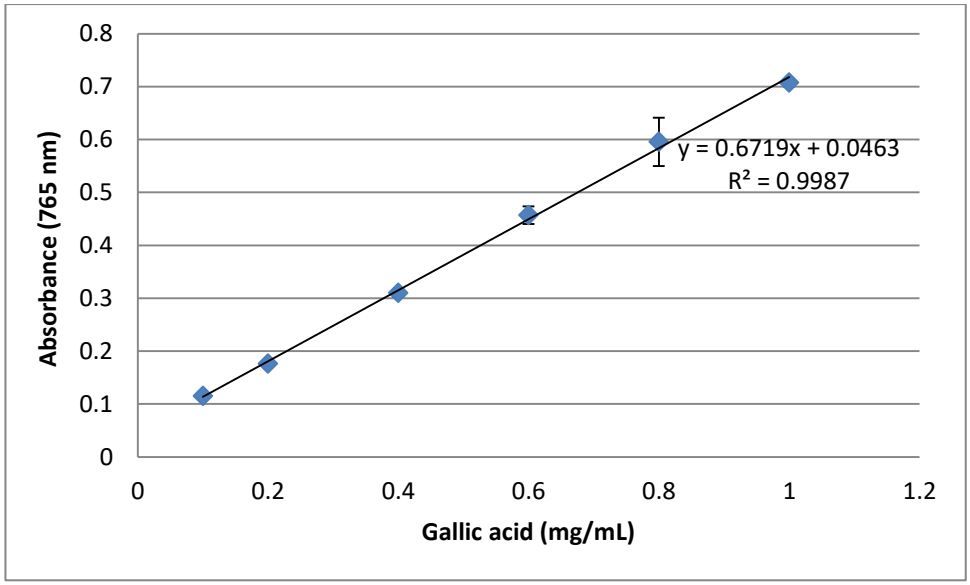
A 6 Normal distribution histogram of mushroom PPO RA residuals



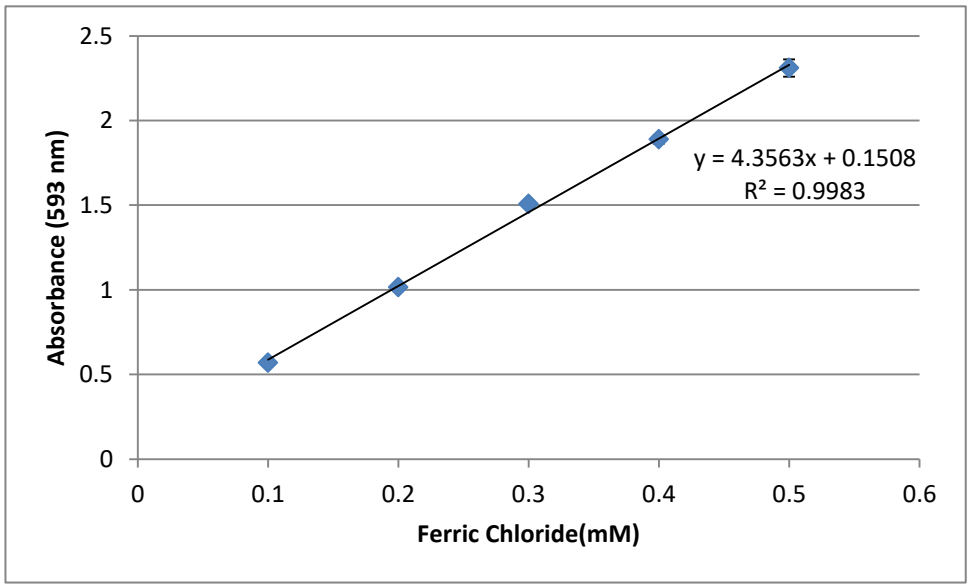
A 7 Graph of linearity of residuals for mushroom PPO RA



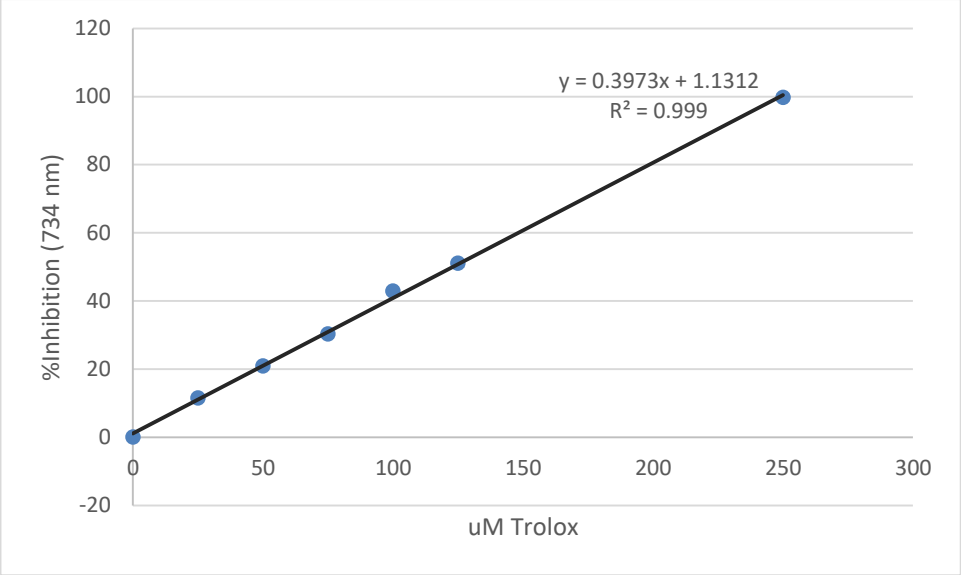
A 8 Graph of independence of residuals for mushroom PPO RA



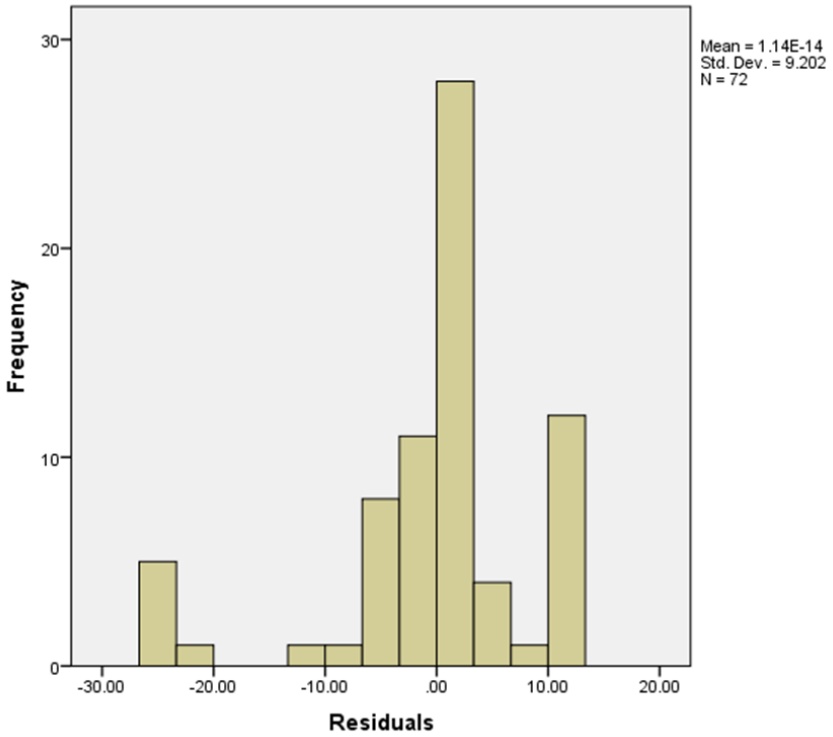
A 9 Calibration curve of gallic acid. Values presented are means (±STDEV)



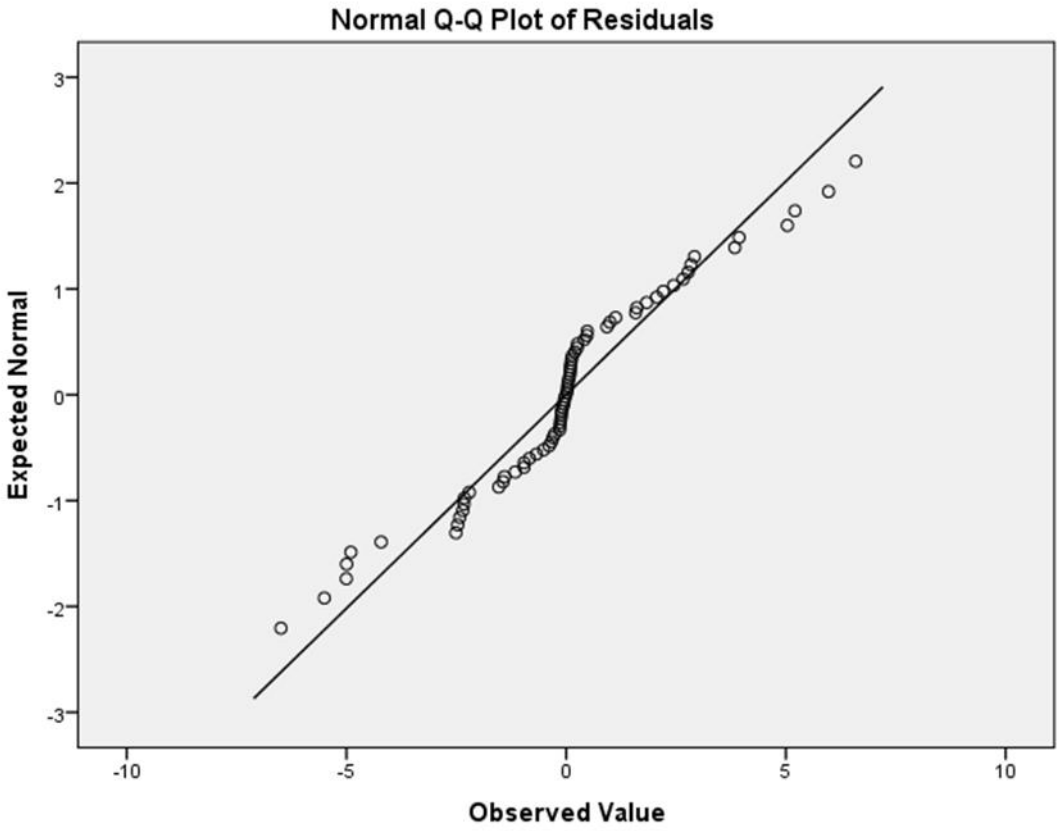
A 10 Calibration curve of ferric chloride. Values presented are means (±STDEV)



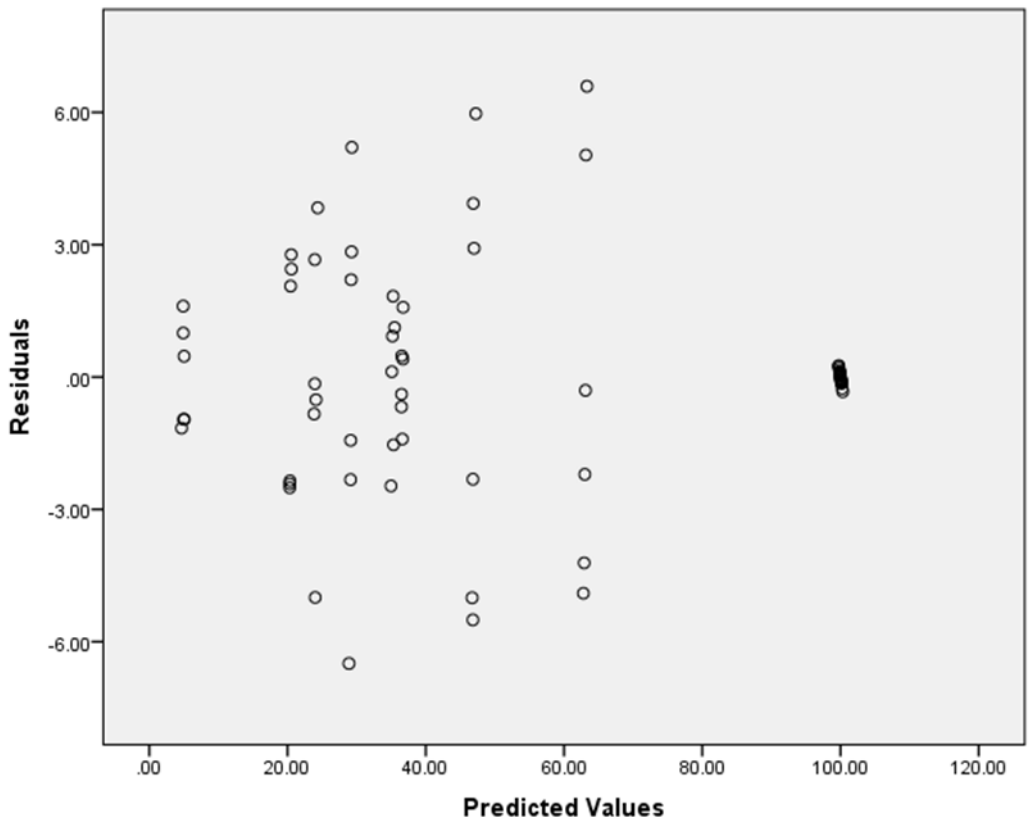
A 11 Calibration curve of Trolox. Values presented are means (±STDEV)



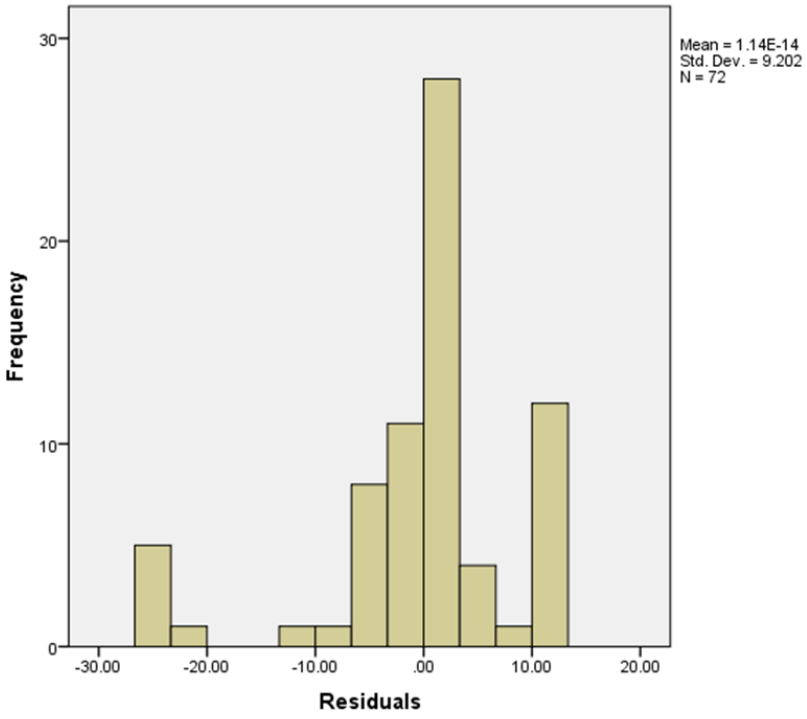
A 12 Normal distribution histogram of strawberry puree POD RA residuals



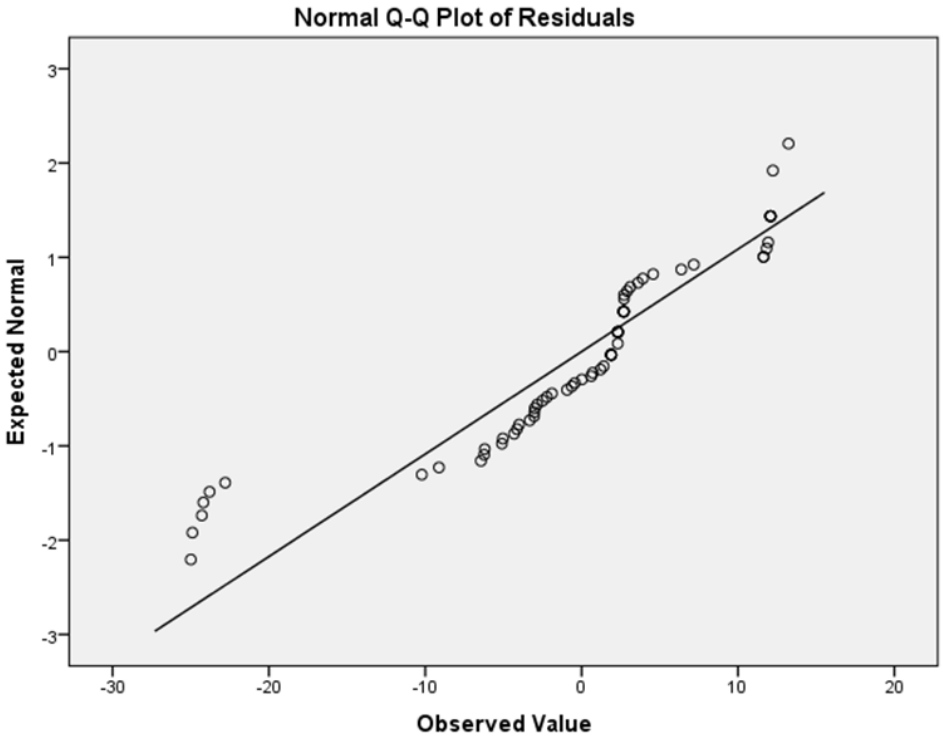
A 13 Graph of linearity of residuals for strawberry puree POD RA



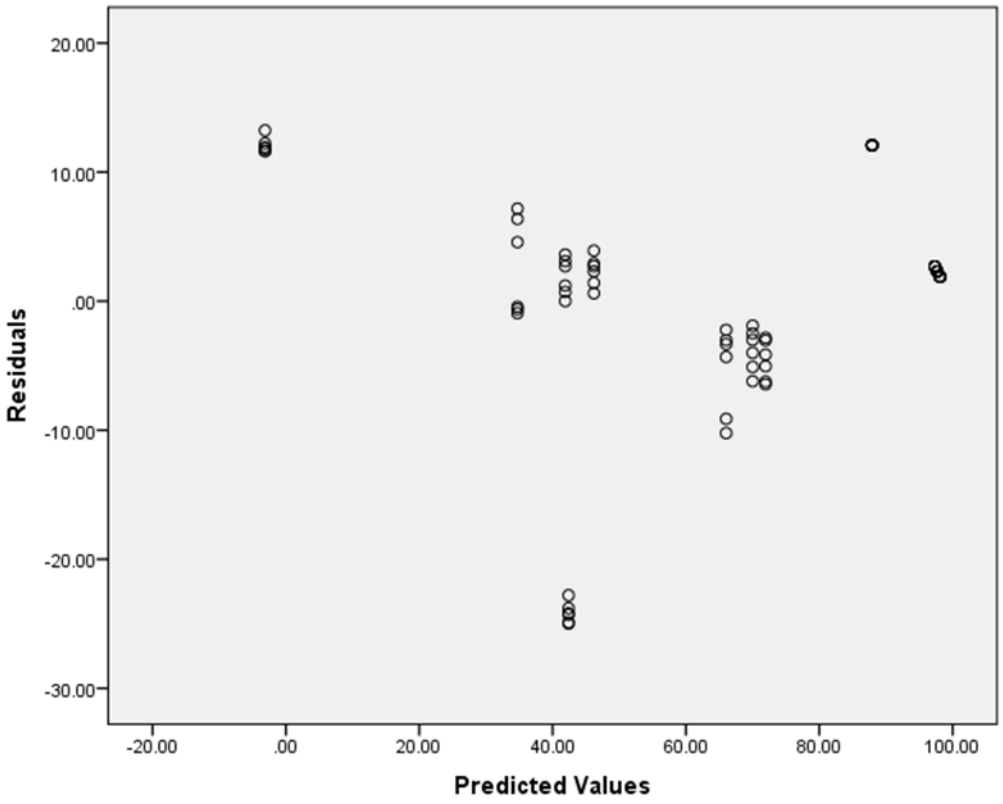
A 14 Graph of independence of residuals for strawberry puree POD RA



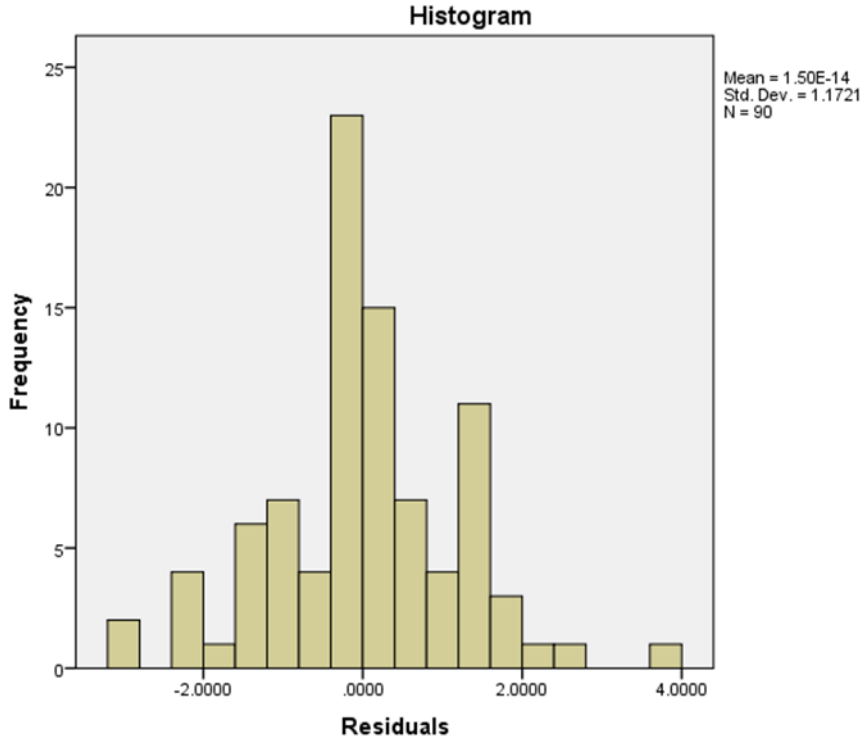
A 15 Normal distribution histogram of strawberry puree PPO RA residuals



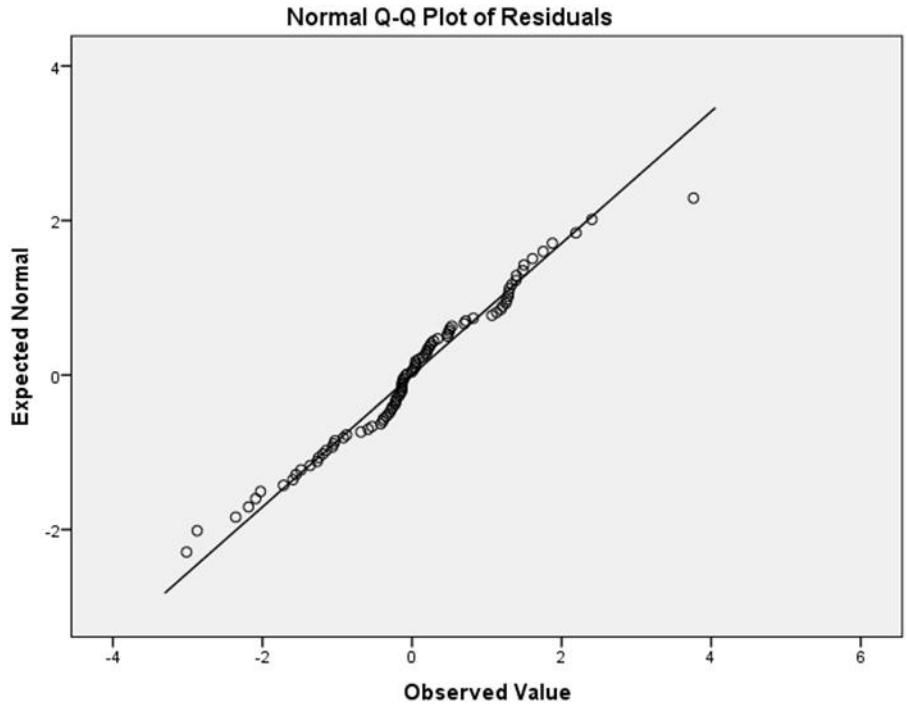
A 16 Graph of linearity of residuals for strawberry puree PPO RA



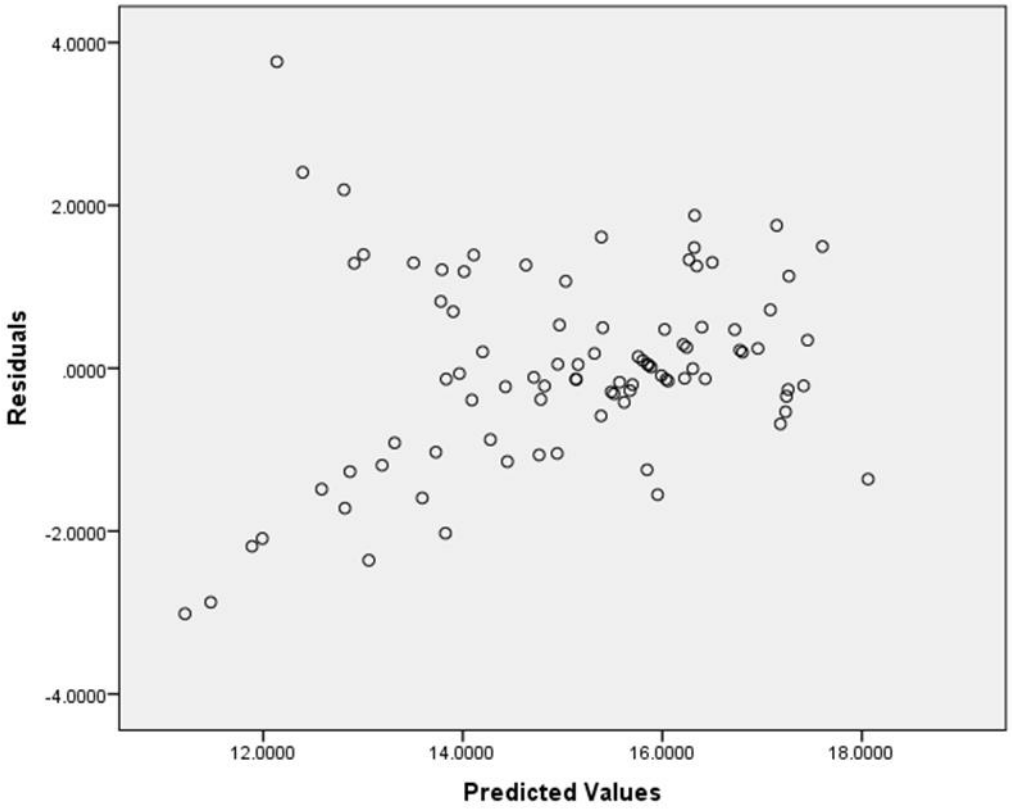
A 17 Graph of independence of residuals for strawberry puree PPO RA



A 18 Normal distribution histogram of strawberry puree TAC residuals



A 19 Graph of linearity of residuals for strawberry puree TAC



A 20 Graph of independence of residuals for strawberry puree TAC

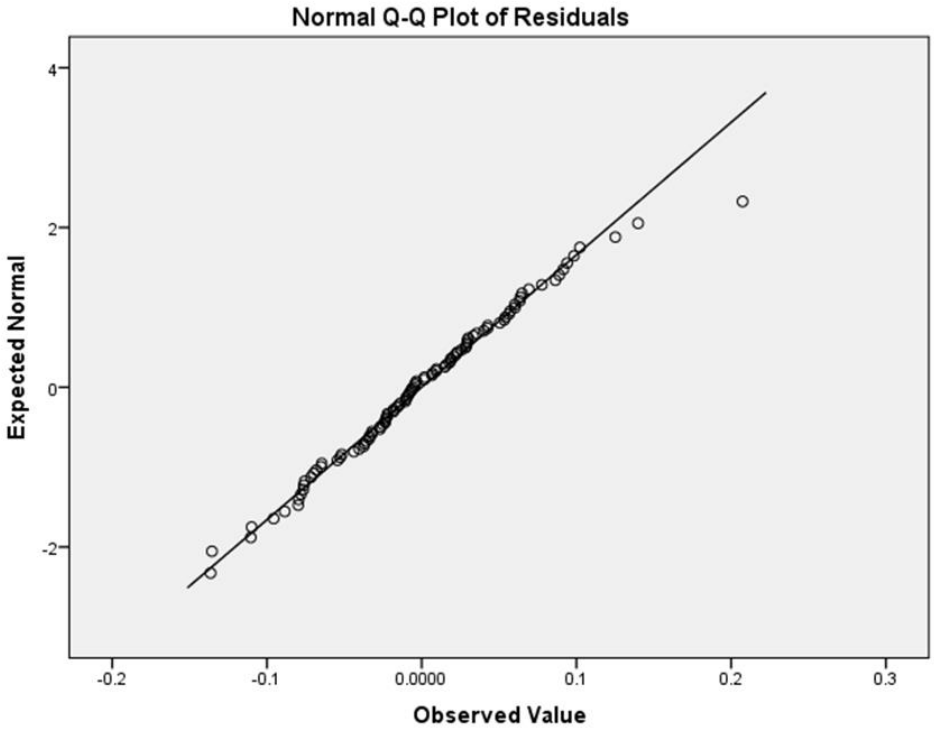
A 21 Results of Shapiro-Wilk test of normality for strawberry puree TPC residuals

Tests of Normality

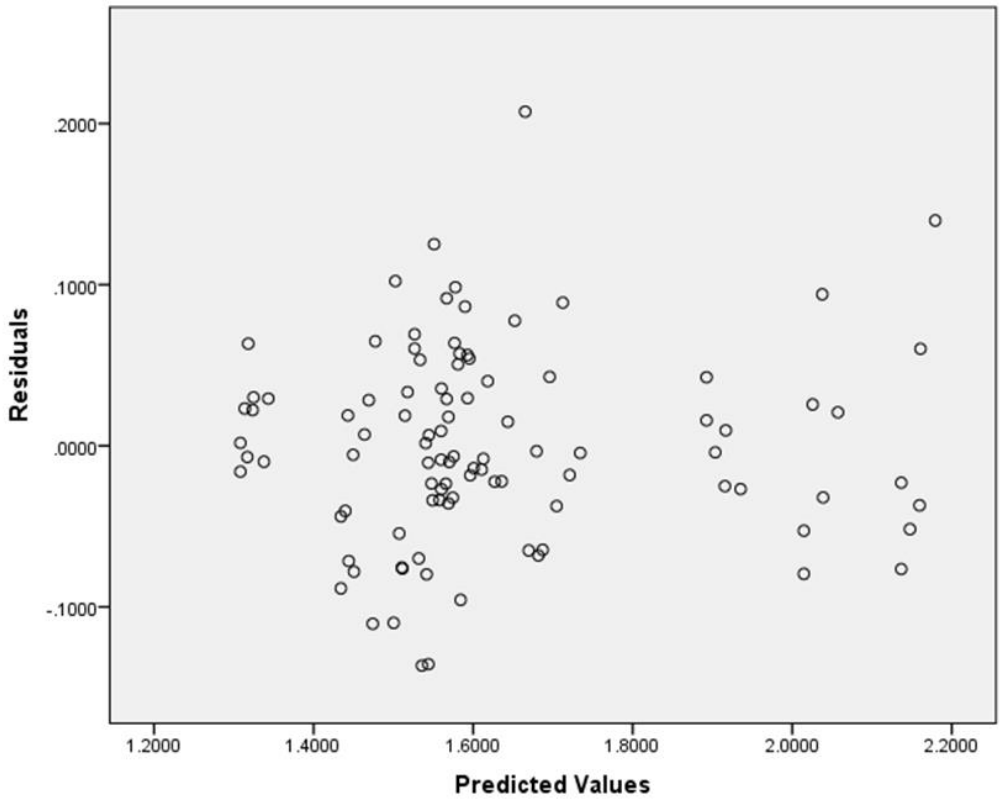
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Residuals	.058	99	.200*	.988	99	.499

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction



A 22 Graph of linearity of residuals for strawberry puree TPC



A 23 Graph of independence of residuals for strawberry puree TPC

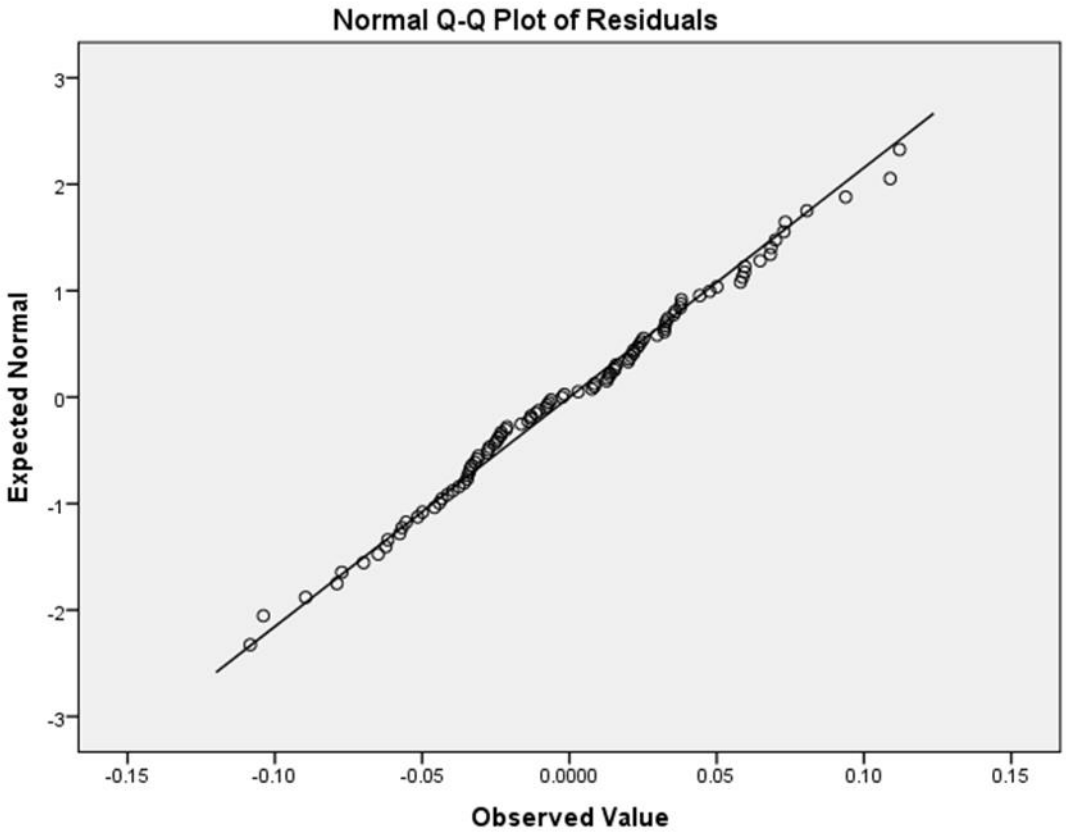
A 24 Results of Shapiro-Wilk test of normality for FRAP analysis of strawberry puree

Tests of Normality

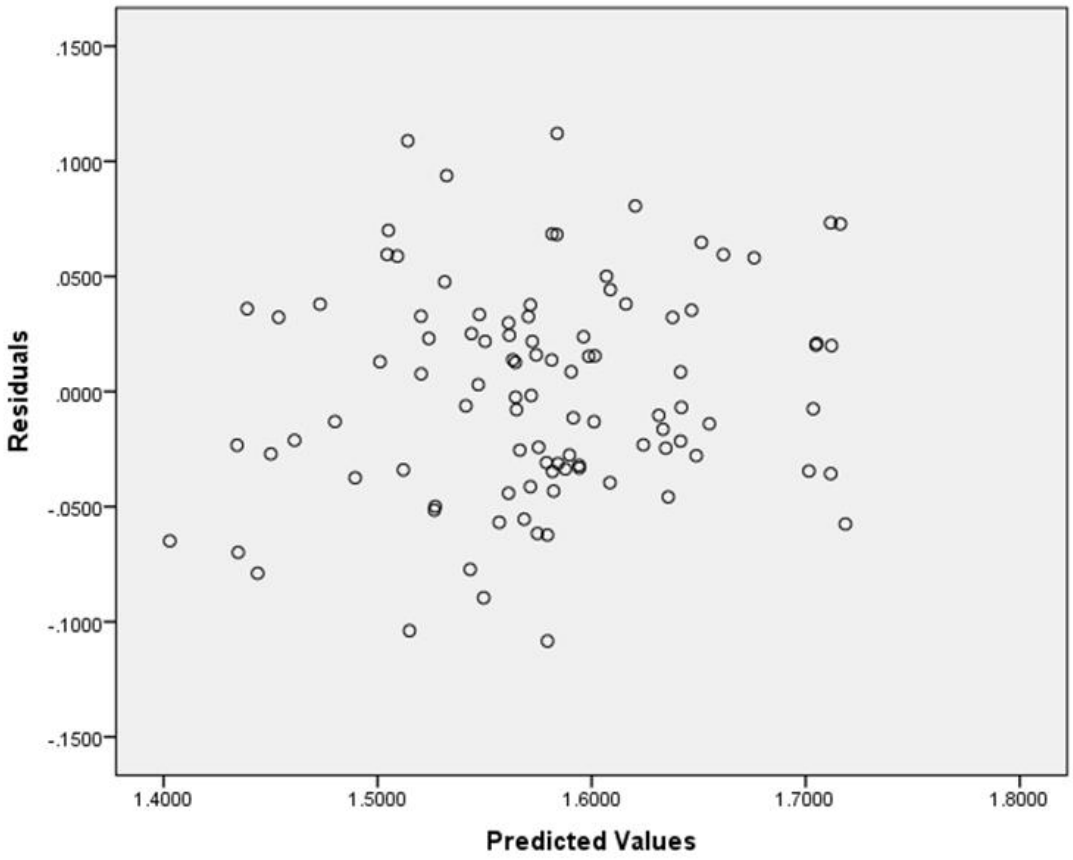
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Residuals	.070	99	.200*	.991	99	.789

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction



A 25 Graph of linearity of residuals for strawberry puree AA as defined by FRAP analysis



A 26 Graph of independence of residuals for strawberry puree AA as measured by FRAP analysis

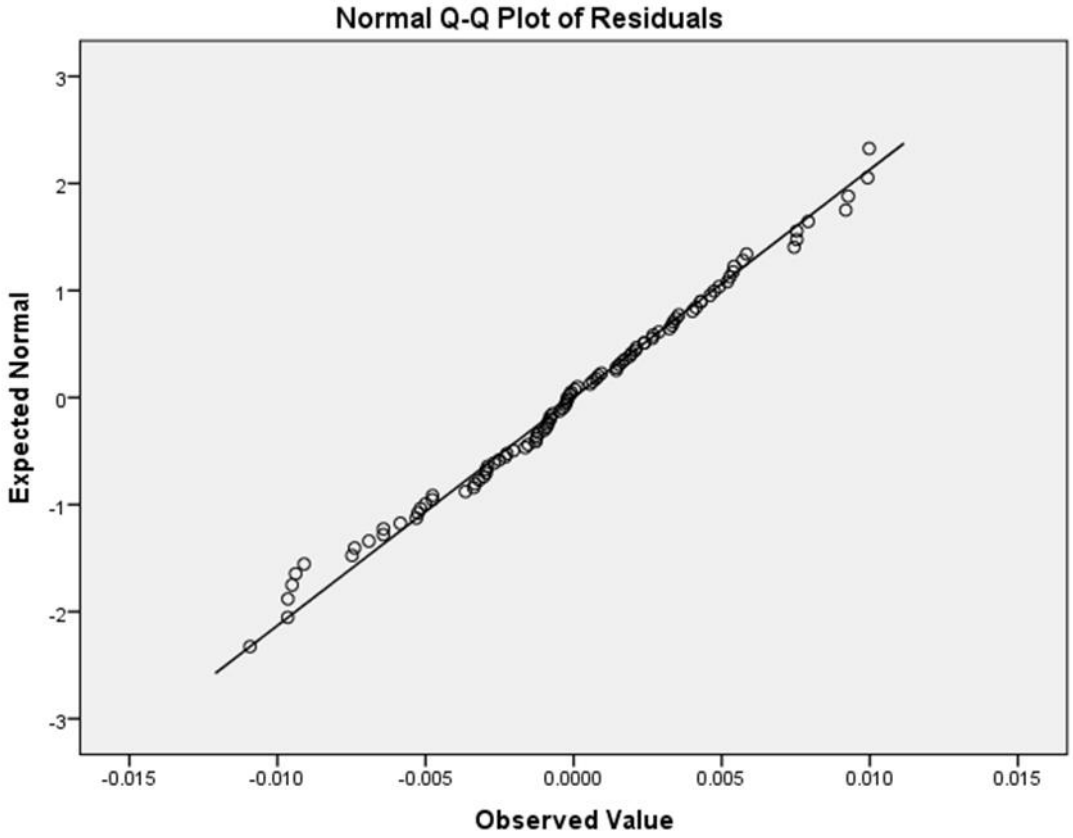
A 27 Results of Shapiro-Wilk test of normality for TEAC analysis of strawberry puree

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Residuals	.059	99	.200*	.988	99	.488

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction



A 28 Graph of linearity of residuals for strawberry puree AA as defined by TEAC analysis

9 Appendix B

List of publications

Tsikrika, K., Chu, B. S., Bremner, D. H. and Lemos, M. A. (2018) 'The effect of different frequencies of ultrasound on the activity of horseradish peroxidase', *LWT - Food Science and Technology*, 89(April 2017), pp. 591–595. doi: 10.1016/j.lwt.2017.11.021.

Tsikrika, K., Lemos, M. A., Chu, B. S., Bremner, D. H. and Hungerford, G. (2017) 'Time-resolved fluorescence observation of di-tyrosine formation in horseradish peroxidase upon ultrasound treatment leading to enzyme inactivation', *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*. Elsevier B.V., 173, pp. 324–327. doi: 10.1016/j.saa.2016.09.035.

List of in progress manuscripts

Tsikrika, Chu, B. S., Bremner, D. H., Hungerford,G. and Lemos, M. A. 'Effect of ultrasonic and thermosonic treatment on the activity of mushroom (*Agaricus bisporus*) polyphenoloxidase and structural changes observation using time-resolved fluorescense'.

Tsikrika, K., Chu, B. S., Bremner, D. H., Savage, A. and Lemos, M. A. 'Effect of Ultrasound on Enzyme Activity and Bioactive Compounds of Strawberry Puree during Storage'.

Conferences

Tsikrika, K., Chu, B. S., Bremner, D. H. and Lemos, M. A. 'Effect of high and low frequency ultrasound on the enzyme activity, bioactive compounds, and antioxidant properties of strawberry juice'. 31st EFFoST International Conference. November 2018, Nantes, France (Accepted Abstract).

Tsikrika, K., Chu, B. S., Bremner, D. H. and Lemos, M. A. 'The effect of Ultrasound on Peroxidase and Polyphenoloxidase Activity'. Abertay Student Conference. June 2017, Dundee, UK.

Tsikrika, K., Lemos, M. A., Chu, B. S., Bremner, D. H. and Hungerford, G., 'Fluorescence as an indicator of HRP and PPO structural change upon application of high frequency ultrasound'. FluoroFest, April 2017. Glasgow, UK.

Tsikrika, K., Lemos, M. A., Chu, B. S., Bremner, D. H. and Hungerford, G. 'Influence of high frequency ultrasound treatment on HRP and PPO structure and activity'. Biophysical Society, 61st Annual Meeting, February 2017. New Orleans, USA.

Tsikrika, Chu, B. S., Bremner, D. H., Hungerford,G. and Lemos, M. A. 'The effect of multi-frequency ultrasound on the activity of peroxidase and polyphenoloxidase'. 30th EFFoST International Conference, November 2016. Vienna, Austria.

Tsikrika, K., Chu, B. S., Bremner, D. H. and Lemos, M. A. 'Effect of Ultrasound on the Activity of Horseradish Peroxidase'. 4th ISEKI_ Food Conference, July 2016. Vienna, Austria.

Tsikrika, K., Chu, B. S., Bremner, D. H. and Lemos, M. A. 'Effect of Multi-frequency Ultrasound on the Activity of Horseradish Peroxidase'. Abertay Student Conference. June 2016. Dundee, UK.

Tsikrika, K., Chu, B. S., Bremner, D. H. and Lemos, M. A. 'Effect of Ultrasound on the Activity of Horseradish Peroxidase'. Early Career Researchers in Food, October 2015. Birmingham, UK.

Tsikrika, K., Chu, B. S., Bremner, D. H. and Lemos, M. A. 'Effect of Ultrasound on the Activity of Horseradish Peroxidase'. Abertay Bioscience Conference, October 2015. Dundee, UK.

Awards

First Prize for Postgraduate Students in Young Scientist of the Year Competition by Institute of Food Science and Technology (IFST), 'The Effect of Ultrasound on the Activity of Horseradish Peroxidase'. April 2016, Dundee, UK.

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